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α-Crystallin-Type Heat Shock Proteins: Socializing Minichaperones in the Context of a Multichaperone Network

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INTRODUCTION

Our knowledge about protein folding has increased substantially over the last 30 years. The traditional view that proteins fold spontaneously (9) was revised upon the finding that many proteins in a living cell will not fold correctly without the assistance of molecular chaperones. Chaperones have been defined as "a family of cellular proteins which mediate the correct folding of other polypeptides, and in some cases their assembly into oligomeric structures, but which are not components of the final functional structures" (75, 76). This definition implies that the predominant function of molecular chaperones is to transiently interact with other proteins, thereby preventing the formation of illegitimate interactions that might otherwise lead to deleterious protein aggregation. Chaperones generally seem to bind to exposed hydrophobic surfaces that will ultimately be buried in the folded state. Controlled release of a substrate protein from the chaperone, often driven by ATP hydrolysis, promotes folding into the native state. Repeated cycles of binding and release may be necessary for productive folding.

Enormous progress has been made in the elucidation of the major chaperones belonging to the Hsp60 (GroEL) and Hsp70 (DnaK) families. A wealth of information on the structural and functional properties of these chaperones has accumulated, and many excellent reviews on this topic are available (18, 40, 111, 196, 251, 257). However, our understanding of other chaperones is comparatively limited. Alternative chaperones are less abundant and less critical for protein folding than are DnaK and GroEL. However, since protein folding has been recognized as one of the central problems in biology, our knowledge about the contribution of minor chaperones to this process is catching up.

The purpose of this review is to draw attention to a family of low-molecular-mass chaperones, the α-crystallin-type proteins. The bulk of our knowledge about this chaperone class comes from studies of the name-giving lenticular α-crystallins. The

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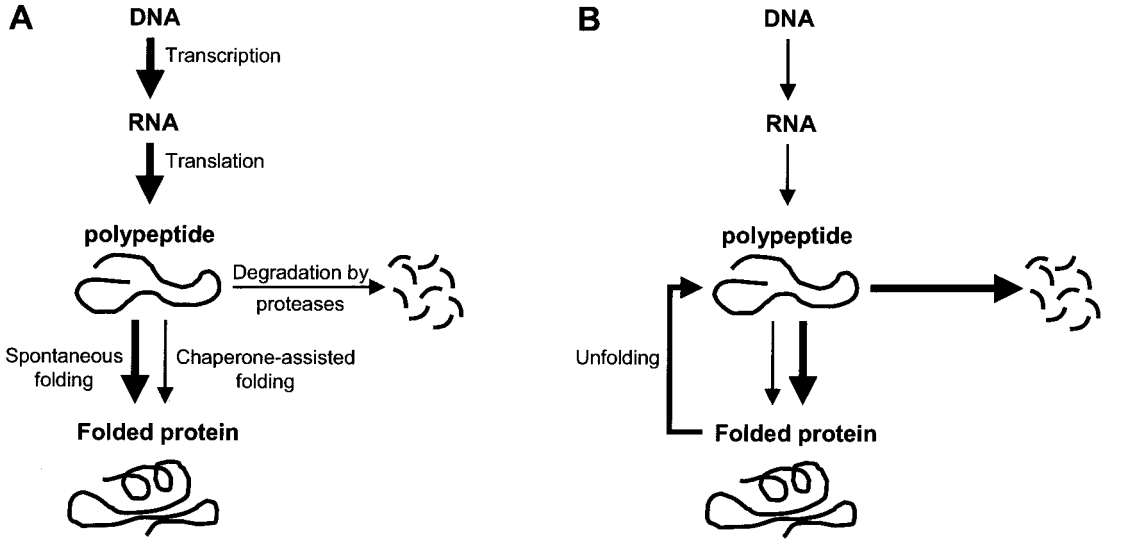

FIG. 1. Simplified model of protein quality control under normal and heat shock conditions. (A) Under optimal growth conditions, the rate of transcription and translation is very high. As indicated by the thick arrow, most proteins fold spontaneously without the assistance of chaperones. Few proteins aggregate and are degraded by proteases. (B) After heat shock, the transcription and translation capacity is reduced. Temperature-induced unfolding returns previously folded proteins to the chaperone-dependent quality control system. Unfoldable proteins are removed by proteases. For simplicity, intermediate steps such as aggregation and disaggregation are not considered in this model.

two forms of α-crystallin,αA- and αB-crystallin, prevent protein precipitation and cataract formation in the vertebrate eye lens (33). Interestingly, α-crystallins not only are present in lenticular tissues but also have been found in many other tissues like heart, brain, and kidney (11). Because of the seminal finding of four α-crystallin-type proteins in *Drosophila* (133), it is well established that α-crystallins comprise a diverse protein family existing in most, but not all, animals, plants, bacteria, and archaea (43, 60, 192, 192a, 220, 349). The presence of α-crystallin-type Hsps in all kingdoms clearly points to important biological functions beyond their specialized role in clear vision. The discovery that α-crystallins can act as molecular chaperones (127, 138) boosted research on this class of stress proteins. The growing interest in this subject is reflected by a rapidly increasing amount of literature, which will be reviewed here.

Most information in this article will be presented from the perspective of a microbiologist. Whenever necessary and appropriate, complementary material on counterparts from higher organisms will be documented. More detailed information on eukaryotic α-crystallins can be found in other articles (11, 62, 71, 193, 268a, 349). The present review first introduces general principles of protein quality control in *Escherichia coli* and then gives details on the regulation, structure, and function of α-crystallin-type proteins. This information will finally be integrated into a model presenting how α-crystallins cooperate in the framework of a complex multichaperone network.

PROTEIN QUALITY CONTROL

Normal Conditions

One of the most critical events in the biogenesis of a protein is the conversion of its linear amino acid sequence into the properly folded three-dimensional structure. As soon as a nascent polypeptide chain emerges from the ribosome, it is prone to misfolding and subsequent aggregation. Although many proteins may fold spontaneously, the initial folding of a significant portion of cellular proteins requires the assistance of molecular chaperones (39, 229). It has been estimated that 20 to 30% of all proteins in the prokaryotic cytoplasm transit through the DnaK or GroEL chaperone machineries before adopting their final conformation (77, 316) (Fig. 1A). Chaperoning occurs both cotranslationally, while the polypeptide is being synthesized, and posttranslationally, after the complete amino acid chain has been released from the ribosome (39). Cotranslational folding requires DnaK and trigger factor, a ribosome-associated protein with peptidylprolyl-*cis-trans* isomerase and chaperone-like activities (66, 316). Both DnaK and GroEL contribute to posttranslational protein folding.

An alternative fate for misfolded proteins is proteolytic degradation. About 20% of all synthesized polypeptides never reach their final destination because fatal errors have occurred during transcription or translation or because correct folding has not been accomplished (357). Before such detrimental proteins accumulate, they are removed by proteases (Fig. 1A). Several universal ATP-dependent protease families have been described (96, 97). Interestingly, the analogy to the major chaperone classes extents beyond their common ATPase activity. Both chaperones and proteases are designed to recognize similar features that are found on unfolded proteins but not on native proteins, namely, surface-exposed hydrophobic patches. This related activity led to the evolution of a similar ring-like architecture of various chaperone and protease complexes (126, 357). Most intriguingly, the proteases involved in protein quality control have intrinsic chaperone activity, either within the same polypeptide (Lon, FtsH, and DegP) or located in associated components of a protease complex (ClpAP and ClpXP, where the ATP-dependent chaperone is underlined). The term "charonin" was invented for this type of multifunctional proteins or protein complexes (97, 272, 338). The tight

combination of chaperone function with proteases suggests that the initial energy-dependent steps of the two processes are similar. Partial unfolding of substrate proteins is most probably the common prerequisite for both activities (304, 313, 351).

Protein synthesis in bacteria is much faster than in eukaryotes. As calculated from a synthesis rate of 15 to 20 amino acids per ribosome per s, *E. coli* produces some 30,000 polypeptides per min (39). This remarkable speed of translation requires a very efficient and accurate protein quality control system. Even if only 30% of the proteins need the attention of chaperones, almost 10,000 polypeptides per min are going through a chaperone cycle. Evidently, the decision of whether a polypeptide can be folded or must be degraded has to be made rapidly. Precise monitoring of the actual state of a protein is required to direct it to the appropriate pathway. It is not clear how the quality control system decides between (re)folding or breakdown when it faces a misfolded protein. Several tagging devices have evolved to sort out the hopeless cases that cannot be rescued by chaperones. Ubiquitinated polypeptides are removed by the proteasome in eucaryotes (47, 340). The N-end rule, variations of which apply to all known organisms, correlates the amino-terminal amino acid with the half-life of a protein (323, 339). Whereas certain residues confer a short-half life, others seem to stabilize a protein. The exposure of a destabilizing residue in damaged proteins directly routes them toward degradation, bypassing any refolding attempts. In *E. coli*, truncated polypeptides that result from abnormally terminated transcription are tagged by the SsrA system and thereby directed toward degradation (150, 329). A recent report demonstrates that abnormal proteins are susceptible to carbonylation, an unrepairable oxidative modification (70). This might provide the signal that a protein is irreparable and therefore destined for degradation. The associated chaperone components of the Clp proteases or the proteasome play a role in the decision whether a polypeptide can be salvaged (97, 357). Although important aspects of protein folding and degradation have been worked out over the last years, many details of the steps between the birth and death of a protein remain to be elucidated. The fate of every single protein in a cell constantly relies on a delicate balance between folding and degradation (121, 357). The rapid biogenesis of proteins in a growing cell puts a high pressure on the quality control machinery even under optimal conditions.

Stress Conditions

Once cellular proteins are folded, various stress conditions pose a serious threat to their integrity. Temperature variations, osmotic changes, antibiotics, solvents, or other chemicals not only interfere with transcription, translation, and protein folding but also often disrupt the faithfully acquired three-dimensional protein structure. The heat shock response elicited by a sudden increase in the ambient temperature is widely used as a model system for studying the impact of stress on biological systems. Proteins whose expression is induced on heat shock are generally called heat shock proteins (Hsps). It is not surprising that the classical chaperones and proteases are among them. All players involved in the regular protein quality control system described in the previous section are required to combat the damage inflicted by a thermal insult. On heat shock, a cell faces numerous problems. Transcription and translation slow because the RNA polymerase subunits RpoA, RpoB, and RpoD (σ^{70}) are thermolabile (23, 211) and because the translation factor EF-G is susceptible to aggregation at elevated temperatures (211) (Fig. 1B). Spontaneous folding of nascent polypeptides at high temperatures is error prone because interactive hydrophobic surfaces become exposed. Even subtle structural changes may suffice to inactivate cellular enzymes. It is evident that protein quality control under such circumstances becomes crucial for sustaining cellular metabolism and viability. In contrast to normal conditions, productive protein folding now occurs predominantly via the chaperone-mediated pathway due to a large number of thermolabile proteins (211). A large portion of already folded proteins gets partially or completely denatured and reenters the quality control system (Fig. 1B). When the rate of denaturation outpaces the refolding capacity, increasing amounts of proteolysis-sensitive substrates are being generated.

The additional demand for folding support during acute stress is met by two strategies: (i) the level of preexisting quality control proteins is elevated; and (ii) additional chaperones that are not expressed or are only weakly expressed under nonstress conditions are induced to counteract severe damage. Members of the first class, which are abundant under all metabolic conditions, are DnaK and GroEL. α-Crystallin-type Hsps usually belong to the second class. Since these proteins are barely expressed at nonstress temperatures in most organisms, the induction factors after a temperature upshift can be very high (several hundred-fold [Table 1]) (258). Proteases are generally less abundant than chaperones regardless of the environmental conditions, indicating that protein refolding is preferred to proteolysis. Transcriptional induction after heat shock of most proteases was measured in the low to intermediate range (between 5- and 30-fold [Table 1]) (258). Although both chaperones and proteases are upregulated under stress conditions, many proteins seem to escape the quality control system and end up in aggregates. Polypeptides trapped in this state have long been considered dead-end products that cannot be rescued. Only recently, it was recognized that certain chaperones are able to solubilize aggregated proteins in vivo. The concerted action of Hsp104, Hsp70, and Hsp40 in yeast (92) or of ClpB and DnaK in *E. coli* (94, 211, 214, 347) recycles precipitated proteins into the folding pathway. Disaggregated material is released in a nonnative form that either can refold spontaneously or can be further processed by cellular chaperones.

In summary, nature has invented an arsenal of powerful strategies to maintain high-quality protein in the cell. Once a nascent polypeptide has folded correctly, it is not left alone. Problems arising within the life span of a protein are solved by chaperones and proteases, which assist in refolding or disposal of nonrefoldable molecules, respectively. The next section will look more closely at the different chaperone families that contribute to protein folding.

MULTIPLE CHAPERONE FAMILIES

Traditionally, Hsps have been grouped into five major families. They were designated Hsp100, Hsp90, Hsp70, Hsp60, and small Hsps according to their molecular masses (37, 40, 213).

TABLE 1. Heat-inducible chaperone and charonin families in *E. coli*

Chaperone family	*E. coli* designation	Molecular mass (kDa)	Heat induction[a]	Cochaperone	Structural features	Demonstrated or suggested chaperone-like function	Reference(s)
Hsp100	ClpB	96	+++		Hexa- or heptameric ring	Solubilization of protein aggregates	94, 153, 369
	ClpA	84	+		Hexameric ring	Protein unfolding for proteolysis	152, 353, 356
	ClpX	46	+		Hexameric ring	Protein unfolding for proteolysis	100, 352
Hsp90	HtpG	71	+++		Dimer	Prevention of protein aggregation	36, 302
Hsp70	DnaK	69	++++	DnaJ, GrpE	Substrate-binding channel	Co- and posttranslational protein folding	39, 368
Hsp60	GroEL	57	+++	GroES	Two heptameric rings	Posttranslational protein folding	39, 360
SC[b] charonin	FtsH (HflB)[c]	71	NI		Multimeric ring	Folding and assembly of membrane proteins	5, 287
	DegP (HtrA)[d]	48	NI		Two hexameric rings	Chaperone at low temperatures	154, 303
	Lon	87	++		Tetramer[e]	Substrate sequestration for proteolysis	335
Hsp33	Hsp33	33	++		Dimer	Protection during oxidative stress	140, 156a, 344a
α-Hsp	IbpA, IbpB	16	+++++		Sphere of 24 subunits[f]	Prevention of protein aggregation	72, 155, 175

[a] Induction factors in *E. coli* after a heat shock from 37 to 50°C as determined by microarray hybridization according to Richmond et al. (258). The induction factors are grouped as follows: +++++, 300-fold; ++++, 50- to 100-fold; +++, 25- to 50-fold; ++, 10- to 25-fold; +, up to 10-fold; NI, not identified in this assay.
[b] SC, single chain
[c] FtsH was identified as σ^{32}-regulated Hsp by Herman et al. (122)
[d] DegP is induced by extracytoplasmic stress, including extreme temperatures, in a σ^{E}- and CpxAR-dependent fashion (102, 208).
[e] The mitochondrial Lon protein from yeast has been shown to be a heptameric ring (306).
[f] Determined for the *Methanococcus jannaschii* Hsp16.5 (155).

Several members of the Hsp100 family are chaperone subunits of protease complexes. Hence, there is good reason to include the single-chain charonins, which bear both chaperone and protease functions in a single polypeptide, in the chaperone list. Hsp33, a novel chaperone whose activity is redox regulated, recently joined the club (140). The updated list of chaperone families therefore comprises seven members (Table 1). It should be noted, however, that more than 30 functionally uncharacterized heat shock genes and proteins have been identified by global transcriptome and proteome analyses in *E. coli* (46, 258, 330). Whatever their function might be, one may anticipate a growing number of chaperone families in the future.

Hsp70 (DnaK) and Hsp60 (GroEL)

The two major chaperone families are Hsp70 and Hsp60. They are also the by far best-characterized chaperones. DnaK and GroEL, the bacterial representatives of these classes, are among the most abundant cellular proteins, indicating their important role in protein folding. In the gram-negative and gram-positive model organisms *E. coli* and *Bacillus subtilis*, respectively, *groEL* is essential whereas *dnaK* is not (80, 183, 240, 271). In both species, serious growth defects and inviability of *dnaK* mutants were observed only at high temperatures (210, 240). In *Streptomyces coelicolor* A3(2), *Pseudomonas syringae* pv. *glycinea*, and *Bradyrhizobium japonicum*, all attempts to construct *dnaK* deletion strains failed suggesting that in some organisms the DnaK function might also be essential under physiological conditions (34, 151, 206). Multiple *hsp70* or *hsp60* genes have been identified in many organisms (83, 189, 273). Often, Hsp70 or Hsp60 homologs are not temperature controlled but are either constitutively expressed or induced by environmental cues other than heat. In some cases, family members seem to be able to functionally replace each other, e.g., the GroEL proteins of *B. japonicum* (84). Other seemingly similar proteins appear to have more specialized functions and are unable to restore the defect caused by a deleted family member, e.g., DnaK and HscA (Hsc66) of *E. coli* (123).

Both major chaperones recognize hydrophobic surfaces of unfolded proteins. To finish the folding job, they require specific cochaperones and ATP (Table 1). The DnaK cycle is driven by the concerted action of DnaJ and GrpE (18, 40). Briefly, DnaJ accelerates the ATPase activity and substrate binding of DnaK whereas GrpE functions as nucleotide exchange factor, promoting the exchange of ADP for ATP. Although details of the GroEL cycle are still disputed, it seems widely accepted that binding of ATP and GroES trigger conformational changes in the GroEL ring and promote folding of the polypeptide captured in its central cavity. Subsequent ATP hydrolysis discharges GroES and the substrate from the complex (40, 111, 126, 257). Among the many details that have been worked out for the Hsp70 and Hsp60 machines are the structural properties not only of the chaperone components but also of their cochaperones (85, 110, 131, 243, 360, 368). GroEL assembles into a cylindrical complex consisting of two homoheptameric rings with a large cylindrical chamber that accomodates substrate proteins (360). Unlike GroEL, DnaK acts as a monomer. There is no high-resolution structure of a full-length Hsp70 protein yet, but the crystal structures of the isolated ATPase and substrate-binding domains have been solved (85, 368). The latter contains a polypeptide-binding channel with a hydrophobic pocket. The completely different architectures of DnaK and GroEL are reflected by different substrate specificities. DnaK binds to short hydrophobic segments of nonnative proteins, whereas the GroEL ring encloses the entire substrate protein. Due to the size limitation of the central cavity, typical GroEL substrates are in the small to medium size range from 20 to 60 kDa (130). DnaK, on the other hand, is able to handle large proteins because it acts on surface-exposed hydrophobic peptide chains (211).

Hsp100

Although DnaK and GroEL are the main actors on the folding stage, there are many other chaperones adding to the fidelity of protein folding. These chaperones have more specialized functions and extend the limits of the DnaK- and GroEL-based folding machineries. With few exceptions to the rule, neither of the alternative chaperones is crucial for survival under physiological growth conditions. Hsp100 proteins are ATP-hydrolyzing chaperones that assemble into ring-shaped structures (100, 152, 369). All members of this family use ATP to promote changes in the folding and assembly of other proteins (269). The subsequent fate of a substrate protein depends on the actual Hsp100 member with which it is interacting (Table 1). ClpB directly binds protein aggregates. ATP-induced structural changes in ClpB are assumed to shear aggregates, preparing them for refolding by the DnaK system (94). ClpA and ClpX represent the substrate recognition subunits of the two-component ClpAP and ClpXP proteases (97, 313, 351). Although much smaller than the other family members, ClpX (46 kDa) is considered as Hsp100 relative. Unlike other family members, ClpX has only one instead of two ATP-binding sites. Polypeptides destined for proteolysis by ClpP are first bound and remodeled by the ATPase components ClpA or ClpX before they are translocated into the heptameric proteolytic ring (135, 254, 291). In the absence of the peptidase subunits, the ATPase oligomer acts as a bona fide chaperone, releasing the substrate in a folding-competent form (352, 356). For completeness, another type of two-component protease, ClpYQ (HslUV), also consisting of an ATPase subunit and a proteolytic component, should be mentioned (209, 260). Although inference from ClpA and ClpX suggests that ClpY (HslU) might possess chaperone activity, this has not been demonstrated yet. The invention of several two-component proteases implies that the intimate association of chaperones together with proteases improves the efficiency of protein degradation by promoting the unfolding of otherwise inaccessible substrate proteins. In fact, it has been shown that ClpP has no proteolytic activity when it is separated from ClpA (132).

Single-Chain Charonins

The same principle applies to single chain charonins, in which chaperone and protease activities are combined on a single polypeptide. It is not trivial to separate the two activities experimentally, but again it seems that chaperone activity might be a prerequisite for efficient proteolysis. Several lines of evidence strongly suggest that the membrane-anchored metalloprotease FtsH has chaperone activity. *E. coli* FtsH binds to denatured proteins and is involved in membrane protein assembly (4, 5). Moreover, growth retardation of an *ftsH* mutant can be partially suppressed by the overexpression of other chaperones (286). Chaperone-like activity has been demonstrated most explicitly for the FtsH yeast homolog Yme1, which directly binds to unfolded polypeptides and suppresses their aggregation (180). A very peculiar protein is the periplasmic serine protease DegP. It undergoes a temperature-dependent activity switch and functions as a chaperone at low temperatures and as a protease at elevated temperatures (303). Whether the activity of other charonins is regulated in a similar temperature-dependent manner remains to be tested. A proteolytically inactive variant of the Lon protease retains its substrate-binding capacity, congruent with a chaperone-like activity (337). Whether active refolding of bound substrates can be achieved is not clear. Direct chaperone activity involved in the assembly of membrane protein complexes has been suggested for the mitochondrial Lon protease (256). It should be pointed out again that the primary function of charonins is not to release substrate proteins for subsequent refolding. Although FtsH, DegP, and Lon, like ClpA and ClpX, sequester other proteins and thereby prevent inappropriate interactions leading to aggregation, the primary purpose of these chaperones is not to rescue other proteins but to prepare them for final destruction.

Hsp90

Three chaperone families remain to be discussed, Hsp90, Hsp33 and α-crystallin (Table 1). Of these proteins, only Hsp90 hydrolyzes ATP, and ATPase activity is essential for chaperone activity in vivo (241). ATP-independent chaperone-like activity can be demonstrated in vitro because Hsp90 bears two chaperone sites, one of which does not require ATP. Hsp90 is essential in yeast and *Drosophila melanogaster* but dispensable in *E. coli* and other bacteria (15, 36). In eukaryotes, Hsp90 is a dedicated chaperone involved in the folding of several signaling molecules including steroid hormone receptors (36, 137). The physiological function of HtpG, the bacterial Hsp90 protein, has not been discerned yet. In vitro, HtpG prevents aggregation of unfolded citrate synthase (CS) by transiently interacting with early unfolding intermediates (139). ATP hydrolysis is not necessary for this activity. A recent report indicates that HtpG has chaperone activity in vivo and is necessary for the optimal folding of certain cytoplasmic proteins in stressed *E. coli* cells (318).

Hsp33

E. coli Hsp33 is at present the only member of a novel redox-regulated chaperone class (140). Database searches suggested, however, that Hsp33 homologs are present in a wide variety of microorganisms. Hsp33 prevents the aggregation of thermally or oxidatively damaged proteins very efficiently in an ATP-independent manner (140). A feature that distinguishes Hsp33 from all other known chaperones is the reversible regulation of its chaperone activity by the redox potential of the cellular environment. Reduced Hsp33 is monomeric and inactive, whereas the oxidized chaperone is dimeric and functional (156a, 344a). The activity switch is brought about by the reversible disulfide bond formation between two pairs of cysteines that coordinate zinc in the reduced state (14). Zinc release and concomitant dimerization of Hsp33 induces structural rearrangements that lead to the formation of potential binding sites for unfolded proteins. Since it is thought that heat shock causes oxidative protein damage (21, 58, 197), it may not be surprising that a redox-sensitive chaperone is under heat shock control.

α-Heat Shock Proteins

The remaining tools in the protein-folding inventory that need to be discussed are the α-crystallin-type Hsps. The widely used nomenclature "small Hsps" for these chaperones is somewhat unfortunate because other Hsps, e.g., Hsp33, GroES, GrpE, and Hsp15 (a ribosome-associated Hsp [161]), are also small heat-inducible proteins but bear no resemblence to α-crystallin. To avoid any confusion, in the remainder of this review the more appropriate term "α-Hsp," as suggested by de Jong et al. (61), will be used for the small Hsps that contain the characteristic α-crystallin domain.

As the (just discarded) designation "small Hsps" indicates, α-Hsps are low-molecular-mass proteins. Their size ranges from 12 to 43 kDa, with the majority being between 14 and 27 kDa. Although these proteins are small, their active entity usually is a large oligomer consisting of multiple subunits. The overall sequence homology between α-Hsps is much lower than in other chaperone classes. A common attribute of all α-Hsps is the presence of a conserved sequence of about 80 amino acids, which is generally referred to as the α-crystallin domain (43). This domain is preceded by an N-terminal region of variable length and considerable sequence diversity. In most cases, a short C-terminal tail extends downstream of the α-crystallin domain (Fig. 2).

During growth under optimal conditions, most bacteria produce either no or negligible amounts of α-Hsps. Under stress conditions, the induction factors both at the mRNA level and at the protein level can be dramatic (13, 115, 142, 178, 205, 220, 263, 276). Microarray-based expression profiling revealed that transcription of *ibpA* and *ibpB*, encoding the *E. coli* α-Hsp members, was heat induced by a factor of 300 (258). This was by far the highest increase among all heat shock genes (Table 1). IbpA and IbpB received their designation of "inclusion body-associated proteins" because they were initially characterized as *E. coli* proteins tightly associated with overexpressed recombinant proteins that had formed insoluble inclusion bodies (6). In general, α-Hsps seem to be dispensable. A temperature-sensitive growth defect of *E. coli ibpAB* mutants is barely detectable but becomes more pronounced in the additional absence of the *dnaK* gene (158, 319). Similarly, disruption of *hsp30* in the fungus *Neurospora crassa* produces no clear phenotype at elevated temperatures but, the *hsp30* mutant was extremely sensitive if heat stress was combined with carbohydrate limitation (248). Deletion of *hsp16.6*, the single α-Hsp gene in the cyanobacterium *Synechocystis* sp. strain PCC 6803, resulted in significantly decreased growth rates and in reduced photosynthetic activity on heat treatment (178). Probably the most drastic phenotype to date has been reported in mice, in which disruption of the eye lens αA-crystallin led to cataract formation due to rapid inclusion body formation (33).

While most chaperones consume energy, α-Hsps are generally believed to be ATP-independent chaperones (138). As a consequence, they lack the refolding capacity of the major chaperones because unfolding and release of folding intermediates cannot be triggered. Nevertheless, α-Hsps have been added to the catalogue of molecular chaperones because they bind to denatured proteins and thereby suppress inappropriate interactions leading to the precipitation of aggregates. The action of α-Hsps maintains substrate proteins in a folding-competent state (72, 175). The reservoir of substrate proteins associated with α-Hsps remains amenable to subsequent refolding by the major chaperone machineries (341). It is important to consider that α-Hsps alone will not efficiently "mediate the correct folding of other polypeptides," according to the chaperone definition given above (75, 76). Only in the context of a multichaperone network are they able to enhance protein folding.

α-HEAT SHOCK PROTEINS IN ARCHAEA, BACTERIA, AND EUCARYA

α-Crystallins are widely distributed among all kingdoms, and their existence has been reported in numerous organisms from bacteria to humans. It is noteworthy, however, that not all organisms contain α-Hsps. Owing to the steadily growing number of whole-genome sequences, it is now possible to determine the exact number of α-Hsp genes in a diverse array of organisms. Table 2 illustrates that this number can vary substantially. With the exception of *Halobacterium* sp. strain NRC-1, whose genome encodes five putative α-Hsps, all other archaea investigated so far have genomes that encode either one or two. The mere presence of these proteins in thermophilic archaea seems puzzling since many of them are devoid of DnaK, DnaJ, and GrpE homologs (192), which are generally believed to be more important for protein folding than are α-Hsps. The universal occurrence of α-crystallins in the archaeal kingdom might be indicative of an early phylogenetic origin of this protein family. Perhaps they were established before the more efficient DnaK machinery was invented. In fact, it has been proposed that archaea that contain *dnaK, dnaJ*, and *grpE* genes acquired them from bacterial donors by lateral gene transfer (99).

Not only thermophilic archaea but also the bacterial thermophiles *Thermotoga maritima* and *Synechococcus vulcanus* each contain a single α-Hsp (Table 2). The *S. vulcanus* protein HspA accumulates after heat treatment (263). Apart from global genome and proteome analyses, detailed information about the heat shock response in thermophilic organisms is scarce. Representative thermophiles from all three phylogenetic domains are capable of mounting a heat shock response (327). It appears that organisms adapted to permanent growth at ferocious temperatures between 60 and 100°C still require responsiveness to temperatures beyond their optimal growth range.

The complete absence of α-crystallin genes in a number of bacteria became evident during mining of genome-sequencing data (Table 2). What do the microorganisms lacking α-Hsps have in common? First, they have rather small genomes, ranging from 0.58 to 2.27 Mb. The 0.58-Mb genome of *Mycoplasma genitalium* demonstrated that the minimal gene set of self-replicating organisms comprises some 470 coding regions (88). As one might predict, a streamlined genome like this devotes only few resources to superfluous functions such as regulation, DNA repair, and protein quality control. The minimal Hsp set consists of GroEL and GroES, DnaK, DnaJ and GrpE, ClpB, Lon, and FtsH. Both *Mycoplasma* species sequenced lack genes coding for ClpA, ClpP, ClpX, HtpG, DegP, and α-Hsps (88, 124). *Treponema pallidum* and *Helicobacter pylori*, with genomes approximately twice and three times the size of the

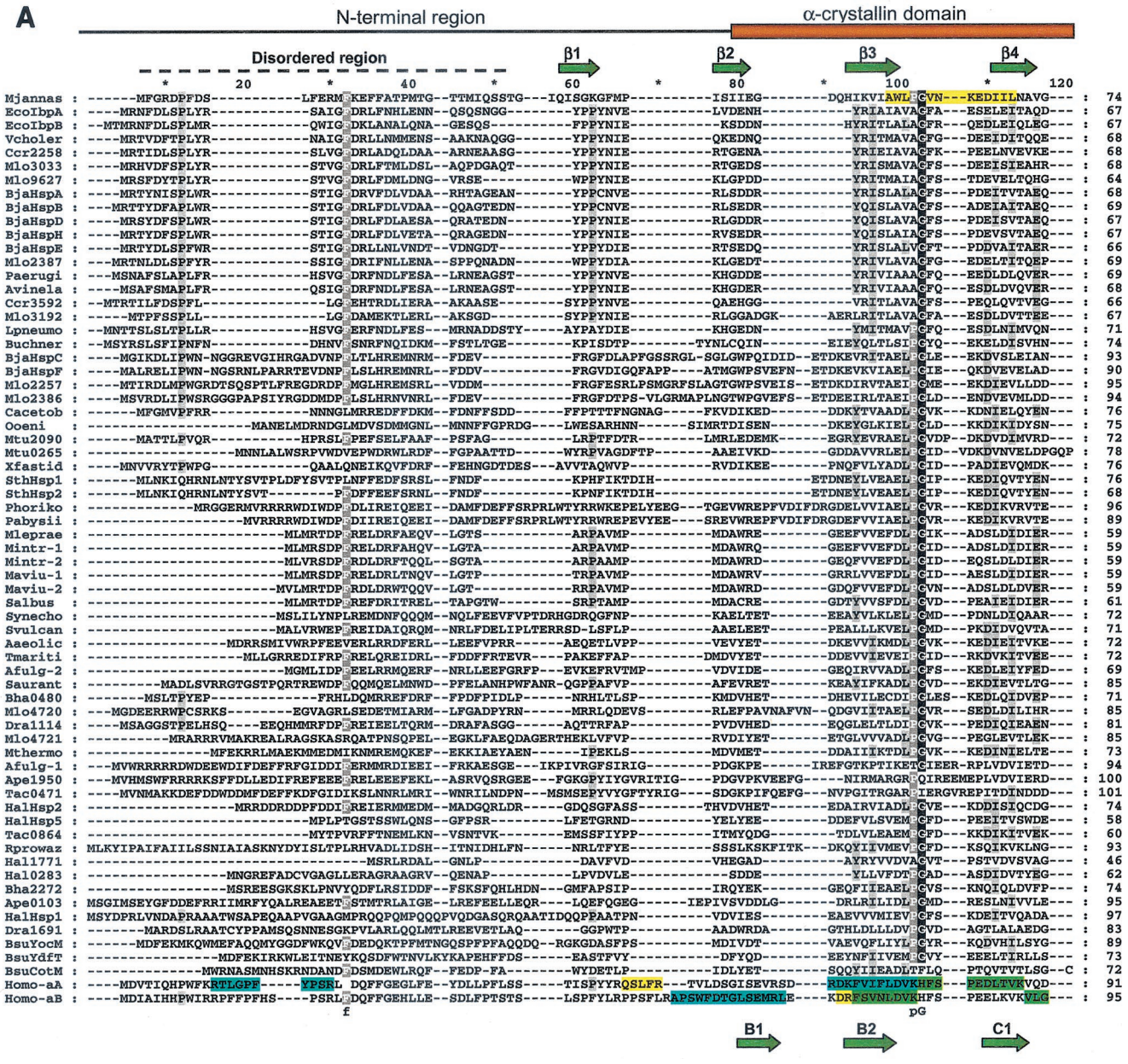

FIG. 2. Amino acid alignment of bacterial and archaeal α-crystallins. Human αA- and αB-crystallins were added for comparison at the bottom. Abbreviations: Mjannas, *M. jannaschii*; Eco, *E. coli*; Vcholer, *V. cholera*; Ccr, *C. crescentus*; Mlo, *M. loti*; Bja, *B. japonicum*; Paerugi, *P. aeruginosa*; Avinela, *A. vinelandii*; Lpneumo, *L. pneumoniae*; Buchner, *Buchnera* sp. strain APS; Caceto, *C. acetobutylicum*; Ooeni, *O. oeni*; Mtu, *M. tuberculosis*; Xfastid, *X. fastidiosa*; Sth, *S. thermophilus*; Phoriko, *P. horikoshii*; Pabysii, *P. abysii*; Mleprae, *M. leprae*; Mintr; *M. intracellulare*; Maviu, *M. avium*; Salbus, *S. albus*; Synecho, *Synechocystis* sp. strain PCC 6803; Svulcan, *S. vulcanus*; Aaeolic, *A. aeolicus*; Tmarit, *T. maritima*; Afulg, *A. fulgidus*; Saurant, *S. aurantiaca*; Bha, *B. halodurans*; Dra, *D. radiodurans*; Mthermo, *M. thermoautotrophicum*; Ape, *A. pernix*; Tac, *T. acidophilum*; Hal, *Halobacterium* sp. NRC-1; Rprowaz, *R. prowazekii*; Bsu, *B. subtilis*; Homo-aA, *Homo sapiens* αA-crystallin; Homo-aB, *Homo sapiens* αB-crystallin. The initial alignment was constructed with CLUSTAL W (322) and then imported into the multiple sequence alignment editor and shading utility GeneDoc (www.psc.edu/biomed/genedoc) and further refined manually. White letters shaded in black or gray indicate amino acids that are identical in at least 80 or 60% of all proteins, respectively. The consensus sequence below the alignment lists these residues in capital and lowercase letters, respectively. Shaded residues printed in black are identical in at least 40% of all sequences. Structural features of *M. jannaschii* Hsp16.5 are depicted on top of the alignment according to crystal structure data (155). For comparison, see Fig. 5B. At the bottom, the secondary-structure assignment of human αA-crystallin is provided (162). α-Crystallin regions that were labeled by bis-ANS or related fluorescent probes are indicated in yellow (280). The highlighted *M. jannaschii* region is equivalent to the labeled peptide of pea Hsp18.1 (175). Segments that cross-linked to target proteins are shaded in blue (278, 281). Peptides identified in the labeling and cross-linking technique are represented in green.

M. genitalium genome, respectively, contain the classical set of Hsps listed in Table 1 with the exception of α-Hsps (89, 324). Since α-Hsps are unable to productively refold denatured proteins, they are probably the chaperones of choice for omission by a cell with a limited genome. In line with the assumption that small genomes go along with the absence of α-Hsps is the presence of multiple α-Hsps in organisms with relatively large genomes such as *B. subtilis*, rhizobia, and eukaryotes (Table 2).

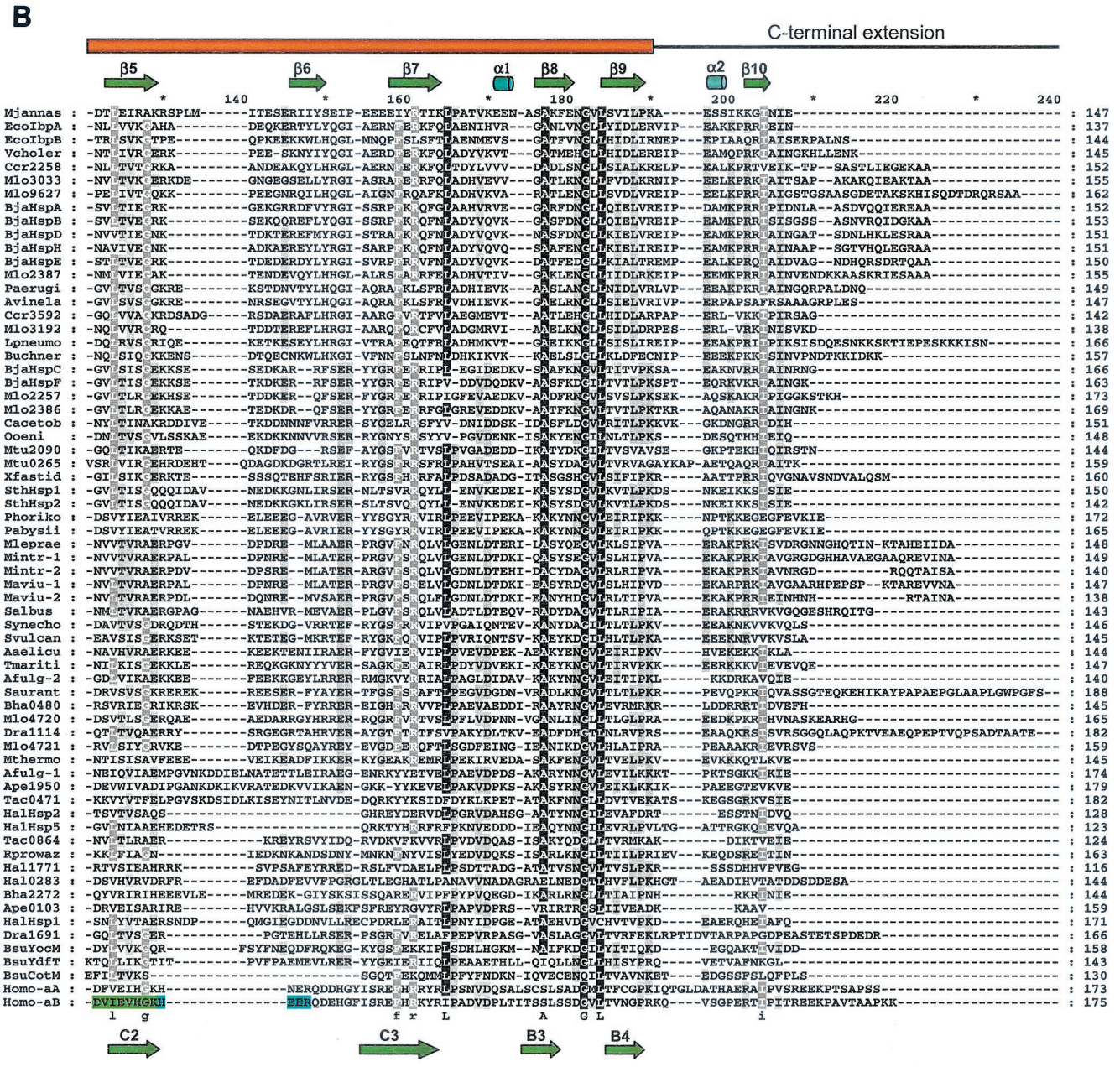

FIG. 2—*Continued.*

The larger the genome, the more genes seem to be devoted to the adaptation to atypical conditions. It should be pointed out, however, that a small genome does not strictly coincide with the absence of α-Hsps. *Buchnera* sp. strain APS (0.64 Mb) and *Rickettsia prowazekii* (1.1 Mb) each carry an α-crystallin-type protein (Table 2). Deducing the absence of these proteins from the limited genome size of these two species would have been misleading.

Why are α-Hsp genes present in some organisms but absent in others? Probably more important than the genome size is the life-style of a bacterium and the ambient temperature it usually faces. *Buchnera* species are obligate endosymbionts of aphids. Their limited gene reservoir renders them completely dependent on a mutualistic relationship with the insect (285). *Rickettsia* species, the living relatives of mitochondria, also are intracellular parasites. Although their life-style resembles that of *Chlamydia* species, rickettsias are normally found in arthropods such as lice and ticks. Only occasionally do they infect humans and cause serious diseases (7). The preference for insects, whose body temperature fluctuates with the ambient temperature, might explain why *Rickettsia* and *Buchnera* species carry an α-Hsp gene in their limited gene set. Most strikingly, all presently known microorganisms without any α-Hsps are parasites and/or pathogens of human and animals. Some of them are degenerated to such an extent that they have become obligate intracellular human parasites, e.g., the *Chlamydia* spe-

TABLE 2. Number of α-crystallins in archaea and bacteria

Organism[a]	Genome size (Mb)	No. of sHsps	Designation(s) and/or (Accession number)	Reference(s)
Archaea				
***Methanococcus jannaschii* DSM2661**	1.66	1	Hsp16.5 or MJ0285 (Q57733)	41
***Methanobacterium thermoautotrophicum* ΔH**	1.75	1	MTH859 (AAB85357)	292
***Pyrococcus horikoshii* OT3**	1.80	1	PH1842 (D71196)	148
***Pyrococcus abyssi* GE5**	1.65	1	PAB2072 (CAB49339)	—[b]
***Aeropyrum pernix* K1**	1.67	2	APE1950 (B72584), APE0103 (B72764)	147
***Archaeoglobus fulgidus* DSM4304**	2.18	2	Hsp20-1 or AF1296 (G69411), Hsp20-2 or AF1971 (B69496)	159
***Thermoplasma acidophilum*[c]**	1.56	2	TA0471 (CAC11613), TA0864 (CAC11993)	264
***Halobacterium* sp. strain NRC-1**	2.57	5	Hsp1 (AAG20020), Hsp2 (AAG18726), Hsp5 (AAG20865), Vng1771c (AAG19995), Vng0283c (AAG18869)	230
Bacteria				
***Borrelia burgdorferi* B31**	1.44	0		87
***Campylobacter jejuni* NCTC 11168**	1.64	0		242
***Chlamydia pneumoniae* AR39**	1.23	0		252
***Chlamydia trachomatis* MoPn**	1.07	0		252
***Haemophilus influenzae* Rd KW20**	1.83	0		86
***Helicobacter pylori* 26695**	1.66	0		324
***Mycoplasma genitalium* G-37**	0.58	0		88
***Mycoplasma pneumoniae* M129**	0.81	0		124
***Neisseria meningitis* MC58**	2.27	0		317
***Treponema pallidum* Nichols**	1.14	0		89
***Ureaplasma urealyticum* serovar 3**	0.75	0		91
***Aquifex aeolicus* VF5**	1.50	1	aq_1283 or HspC (A70411)	60
***Buchnera* sp. strain APS**	0.64	1	IbpA (BAB13269)	285
Clostridium acetobutylicum	4.13	1	Hsp18 (A40592)	235a
***Mycobacterium leprae* TN**	3.27	1	Hsp16.7 (P12809)	52
***Pseudomonas aeruginosa* PA01**	6.30	1	IbpA (AAG06514)	308
***Rickettsia prowazekii* Madrid E**	1.11	1	Hsp22 (CAA14735)	7
***Synechocystis* sp. strain PCC 6803**	3.57	1	SP21 or Hsp17 (S74956)	144
***Thermotoga maritima* MSB8**	1.80	1	TM0374 (D72385)	227
***Vibrio cholerae* N16961**	4.0	1	HspA (AAF93196)	117
***Xylella fastidiosa* 9a5c**	2.68	1	XF2234 (AAF85033)	289
Azotobacter vinelandii		≥1	HspB (P96193)	195
Legionella pneumophila		≥1	GspA (S49042)	2
Oenococcus oeni Lo8413		≥1	Hsp18 (CAA67831)	142
Stigmatella aurantiaca DW4/3-1		≥1	SP21 or HspA (A49942)	115
Streptomyces albus G		≥1	Hsp18 (Q53595)	275
Synechococcus vulcanus		≥1	HspA (BAA32501)	263
***Bacillus halodurans* C-125**	4.2	2	BH2272 (BAB05991), BH0480 (BAB04199)	315
Caulobacter crescentus	4.02	2	CC2258 (AAK24229), CC3592 (AAK25554)	233
***Deinococcus radiodurans* R1**	3.28	2	DR1114 (A75436), DR1691 (A75367)	355
***Escherichia coli* K-12 MG1655**	4.60	2	IbpA (P29209), IbpB (G65170)	24
***Mycobacterium tuberculosis* H37Rv**	4.40	2	Hsp16.3, Acr or MT2090 (P30223), MT0265 [G70939]	51
Mycobacterium avium		≥2	18-kDa antigen 1 (P46729) 18-kDa antigen 2 (P46731)	29
Mycobacterium intracellulare		≥2	18-kDa antigen 1 (P46730) 18-kDa antigen 2 (P46732)	29
Rhizobium tropici CIAT899[d]		≥2		205
Streptococcus thermophilus NDI-6		≥2	Hsp1 (AAC64906), Hsp2 (AAC64908)	239
***Bacillus subtilis* 168**	4.20	3	YocM (BG13526), YdfT (BG12167), CotM (BG11822)	167
Bradyrhizobium sp. (*Lupinus*)[d]		≥3		220
Rhizobium leguminosarum bv. viciae[d]		≥4		220
Sinorhizobium meliloti	6.68	5	SMa1118 (NP_435851), SMb21294 (NP_437338), SMb21295 (NP_437339), SMc04040 (NP_386929), SMc01106 (NP_384520)	89a
Rhizobium sp. strain NGR234[e]		≥5	$HspA_N$, $HspB_N$, $HspC_N$, $HspD_N$, $HspE_N$	220, 235
Rhizobium etli 8002[d]		≥5		220
Rhizobium leguminosarum bv. trifolii[d]		≥6		220
***Mesorhizobium loti* MAFF303099**	7.60	8	mll2257 (NP_103645), mll2386 (NP_103744), mll2387 (NP_103745), mll3033 (NP_104232), mlr3192 (NP_104351), mlr4720 (NP_105524), mlr4721 (NP_105525), mll9627 (NP_109478)	143
Rhizobium etli CNPAF512[d]		≥8		205
Bradyrhizobium japonicum 110 *spc4*[e]	8.7	≥10	HspA (P70917), HspB (P70918), HspC (AAC44757), HspD (O69241), HspE (069242), HspF (CAA05837), HspH (O86110)	219, 220, 224, 225
Bradyrhizobium sp. (*Parasponia*)[e]		≥10	$HspA_P$, $HspB_P$, $HspC_P$, $HspD_P$	220, 235

[a] Organisms whose genome has been sequenced completely are shown in bold type.
[b] Information found at the Genoscope website (http://www.genoscope.cns.fr).
[c] The carboxy-terminal end of a 44-kDa protein (Ta0437 in reference 264 [accession no. CAC11579]) displays very limited similarity to the C terminus of α-crystallin domains. Very pronounced similarity of the entire protein in both molecular mass and sequence to a large number of ArsA proteins suggests that it functions as arsenite-translocating ATPase rather than as an α-crystallin-like chaperone.
[d] As determined by two-dimensional gel analysis.
[e] As determined by a combination of two-dimensional gel analysis and cloning of α-Hsp encoding genes.

cies (252). In their more or less isothermal niche, the mammalian host, an elaborate system responding to temperature fluctuations might become obsolete. Incidentally, the absence of α-Hsps goes along with the absence of cold shock proteins (CSPs) in most cases. The only exception, *Haemophilus influenzae*, encodes just a single CSP, whereas α-Hsp-containing organisms, such as *E. coli, B. subtilis*, and *Lactobacillus lactis*, generally produce multiple CSPs during cold stress (98, 361). CSPs function as RNA chaperones, facilitating the translation of mRNA that tends to form secondary structures after a temperature downshift. Assuming that the simultaneous lack of α-Hsps and CSPs is no coincidence, it seems as if living inside mammalian hosts poses few thermal challenges and therefore obliterates the need for sophisticated temperature-responsive systems.

B. subtilis, Halobacterium sp. strain NRC-1, and rhizobial species are exceptional among bacteria and archaea in that they encode more than two α-Hsps (Table 2). Of the three putative α-crystallin-type proteins of *B. subtilis*, only YocM and CotM were recognized as family members during the initial open reading frame annotation (167). Like many potential α-crystallins in other annotated genome sequences, *B. subtilis* YdfT was categorized as a protein with unknown function. In fact, the similarity of CotM, YocM, and YdfT to other α-crystallins is low (Fig. 2). CotM is a dedicated α-crystallin involved in the assembly of the outer spore coat (120). Evidence for a chaperone-like activity of CotM was not found. CotM, YdfT, and YocM all might have rather specialized functions that do not require all conserved α-crystallin residues.

Multiple α-Hsps were detected during proteome analysis of various plant root-nodulating bacteria (205, 220, 226). A more detailed investigation of *B. japonicum* extracts uncovered at least 10 heat-induced small proteins belonging to the α-crystallin family (219). On the basis of their primary amino acid sequence, the α-Hsps were tentatively divided into two classes, class A and class B (220). Class A proteins (HspA, HspB, HspD, HspE, and HspH) are closely related to the *E. coli* IbpA and IbpB proteins (Fig. 2). Class B α-Hsps (HspC and HspF) are distinguished from class A proteins by their much longer N-terminal region and a shorter C-terminal extension. They have little similarity to *E. coli* α-Hsps. The presence of two α-Hsp classes was also found in *Bradyrhizobium* sp. (*Parasponia*) and in *Rhizobium* sp. strain NGR234 (235). Additional evidence for the presence of multiple α-Hsps and for the existence of distinct classes in rhizobia was provided by the determination of the entire genome sequence of *Mesorhizobium loti* and *Sinorhizobium meliloti* (89, 143). Eight α-Hsp genes encoding four class A and four class B proteins were identified in the first organism, and five genes encoding three class A and two class B proteins were identified in the latter. In this respect, the situation in rhizobial cells is similar to that in plant cytosol, in which three classes of α-Hsps (class I, II, and III) have been described (268a, 349).

α-Hsps are not the only rhizobial chaperones that are present in multiple copies. Several *groESL* operons or *groEL* genes were found not only in *Rhizobium leguminosarum, S. meliloti, B. japonicum*, and *M. loti* but also in many other microorganisms (83, 105, 144, 238, 345). What could be the underlying rationale for the evolution of such multigene families? At least four different reasons can be envisaged. (i) Simultaneous induction of redundant copies of a stress gene rapidly elevates the cellular pool of that protective protein class. Such a coordinated parallel induction of α-Hsps was observed in *B. japonicum* (220). (ii) Providing individual family members with different promoter sequences permits induction in response to a variety of stimuli, a strategy used in the case of rhizobial *groESL* operons (12, 83). (iii) A multigene family relieves the individual genes from functional constraints. Therefore, each gene is free to evolve, allowing it to acquire new functions. The *B. subtilis* CotM protein, for example, might be the product of such mutational diversification resulting in its role in sporulation. (iv) Given that a protein functions as an oligomer (as GroEL and α-Hsps do), slightly different versions of that protein might assemble into hetero-oligomers. Mixed oligomers probably possess substrate specificities or functions slightly different from homo-oligomeric complexes (312). Whatever the exact reason for a gene family might be, it is easily conceivable that iterated stress genes allow a more flexible response to changing conditions than a single gene.

Eukaryotes are well known for containing multiple α-crystallins. *Saccharomyces cerevisiae* inhabits the lower end of the scale with two α-Hsps (Hsp26 and Hsp42) (93), whereas plants lie at the upper end. The *Arabidopsis thaliana* genome exodes 19 α-Hsps. Six families of plant α-Hsps can be distinguished based on their sequence similarities and their cellular localization. Three families (I, II, and III) reside in the cytosol; one is localized to the chloroplasts, one is localized to the mitochondria, and one is localized to the endoplasmic reticulum (268a, 348, 349). Nine human α-Hsps, including αA- and αB-crystallin, have been described (144). Once thought to be eye lens-specific proteins, mammalian α-crystallins have now been found in many cell types (11, 193). In contrast to plant α-Hsps, the mammalian family members seem to be restricted to the cytosol and to the nucleus and do not localize to other cellular compartments.

SEQUENCE DIVERGENCE OF ARCHAEAL AND BACTERIAL α-CRYSTALLINS

Members of the α-Hsp superfamily consist of three regions, a characteristic α-crystallin domain flanked by a poorly conserved N-terminal region and a short C-terminal extension (43, 61). Extensive alignments and phylogenetic analyses of plant and animal α-Hsps can be found elsewhere (43, 61, 268a, 348–350). An overall alignment of 65 α-Hsps from archaea and bacteria reveals only very few highly conserved positions (Fig. 2). The prototype α-crystallins, humanαA- and αB-crystallin, are listed for comparison. The sequence comparison clearly demonstrates that α-Hsps are related but quite distinct. Pairwise alignments between *Methanococcus jannaschii* Hsp16.5, *E. coli* IbpA, or *B. subtilis* CotM and human αA-crystallin show that the sequence identity is as low as 23, 21, and 19%, respectively. Closely related proteins like *E. coli* IbpA and IbpB or human αA- and αB-crystallins have not more than 52 or 58% identical amino acids. Rhizobial α-Hsps show the highest degree of sequence similarity, provided that they belong to the same class. For example, 74% the amino acids in *B. japonicum* HspA and HspB (class A) and 61% of those in HspC and HspF (class B) are identical.

As can be seen in Fig. 2, not a single amino acid is entirely

conserved throughout all the proteins examined. Only five residues (corresponding to G62, L111, A122, G127, and L129 of *M. jannaschii* Hsp16.5) are present in more than 80% of all sequences. The last three residues occur in an A-x-x-x-n-G-v-L consensus motif toward the end of the α-crystallin domain; this motif is the most significant indicator of the domain (43, 61, 349). Almost universally conserved are the G127 and L129 residues, which occur in all but one protein. In CotM from *B. subtilis*, the residue equivalent to G127 is a glutamine. In addition, CotM carries replacements of other conserved residues (e.g., the amino acids equivalent to P61 and G62 in Hsp16.5 are lysine and histidine in CotM). Probably owing to this diversification, CotM has no discernible chaperone activity and serves a unique function in spore coat formation (120). Hsp1 from *Halobacterium* sp. strain NRC-1 contains a cysteine instead of the conserved leucine at position 129. As for most of the sequences listed in Table 2, nothing is known about the functional role of this putative protein. It is possible that Hsp1, like CotM, has structural and functional properties different from standard α-Hsps.

All five highly conserved amino acids reside in the α-crystallin domain, which carries a few additional moderately conserved residues. The flanking regions are even more divergent. Large deviations in sequence and length are evident in Fig. 2. An alignment can be enforced only at the expense of numerous gaps, in particular in the N-terminal region. Very few residues are retained in more than 40% of all sequences. The only exceptions are two characteristic isoleucines (I144 and I146 in Hsp16.5) in the C-terminal extension. They are separated by a single variant amino acid and play an important structural and functional role, at least in *M. jannaschii* Hsp16.5, wheat Hsp16.9, and both classes of *B. japonicum* α-Hsps (see below) 155, 338a; S. Studer, M. Obrist, N. Lentze, and F. Narberhaus, submitted for publication).

REGULATION OF α-HEAT SHOCK PROTEINS

As indicated in the quality control section (see above), it is important that the cellular level of chaperones and proteases be adjusted to the prevailing environmental conditions. The cellular level of chaperones and proteases must be strictly controlled because both excessive and limited amounts compromise the fitness of a cell. The regulatory mechanisms controlling their expression are very diverse. Regulation in most cells occurs at the level of transcription. In eukaryotes, elevated expression of heat shock genes is accomplished by transcriptional activation. With some modifications to the theme, the general principle is conserved in yeast, vertebrates, and plants. In brief, an inert non-DNA-binding heat shock transcription factor (HSF) is converted upon heat shock to a transcription-competent activator that binds to a *cis*-acting heat shock element (HSE) in the promoter region of heat shock genes (212, 236). The activation process of the HSF goes along with its trimerization and phosphorylation. Stress-induced molecular chaperones, most importantly Hsp70, autoregulate the heat shock response by direct binding to the activation domain of HSF (212, 284).

Heat shock regulation in archaea is largely unsolved. The archaeal proteins involved in basal transcription are related to those of the eukaryotic transcription machinery (19, 179, 300). However, archaea have neither an identifiable HSF nor an HSE in heat shock gene promoter regions (192). Bacterial heat shock promoters or regulatory elements are also absent in archaeal genomes. Transcription of the heat-inducible *cct*1 (chaperonin-containing Tcp-1) gene in the archaebacterium *Haloferax volcanii* requires only sequences within the core promoter region (320). The finding of multiple variants of the promoter-binding TATA-binding protein led to the hypothesis that alternative forms of TATA-binding protein are responsible for transcription under heat shock conditions (321).

The latter strategy is strikingly reminiscent of heat shock regulation in *E. coli*. It uses alternative transcription factors, so-called sigma factors, to modulate heat shock gene expression. Bacterial sigma factors are subunits of the RNA polymerase that confer promoter specificity to the core enzyme (103, 134, 358). Specific transcription of the majority of heat shock genes in *E. coli* depends on the sigma factor σ^{32} (RpoH) (102). The cellular concentration of σ^{32} increases transiently after a temperature upshift, primarily as the result of elevated translation of *rpoH* mRNA and of increased stability of the sigma factor. At normal temperatures, σ^{32} is rapidly degraded by FtsH. The requirement of DnaKJ for σ^{32} proteolysis couples the availability of chaperones to heat shock gene transcription and provides a homeostatic heat shock control mechanism (38, 102, 366). The *ibpAB* operon, which encodes both α-Hsps of *E. coli*, belongs to the σ^{32} regulon (6, 46) (Fig. 3A). Immunodetectable levels of IbpA and IbpB proteins in *rpoH* mutants suggest, however, that a σ^{32}-independent pathway participates in the expression of these chaperones (6, 170). Recently, it was found that *ibpB* alone can be transcribed from a σ^{54} (RpoN)-dependent promoter (164a).

Regulation by alternative sigma factors has also been described for two other bacterial α-Hsp genes (Fig. 3A). *Legionella pneumophila gspA* is transcribed from a σ^{70}-type and a σ^{32}-type promoter (2, 3). Induction of *gspA* expression by stress stimuli and during intracellular infection occurs predominantly via the latter promoter. Hsp16.3 of *Mycobacterium tuberculosis* is, at least in part, dependent on SigF, one of many stress-responsive sigma factors in this organism (194). Hsp16.3 (Acr) is not heat shock responsive but accumulates in the transition to stationary phase, during hypoxia and infection of macrophages (364, 365). Many conditions that enhance the SigF level have no effect on *hsp16.3* expression, indicating that SigF is not the sole regulator of this gene. The involvement of the two-component regulatory pair Rv3133c/Rv3132c has recently been inferred from a microarray approach (283).

During investigation of other heat shock regimes, it turned out that positive control by alternative sigma factors is not typical of many other bacteria. Rather, transcriptional repression of heat shock genes is a widespread strategy found in many phylogenetically distinct bacteria. Overlapping or nearby repressor and RNA polymerase-binding sites prevent transcriptional initiation under circumstances where the repressor is competent for DNA binding. A number of heat shock gene repressors have been identified recently (114, 222). Some of them also control α-Hsp genes (Fig. 3B). An intriguingly simple mechanism was discovered in *Streptomyces albus*. At low temperatures, RheA (for "repressor of hsp eighteen"; formerly OrfY) inhibits the transcription of *hsp18* (276, 277). As the result of a thermally induced conformational change, the re-

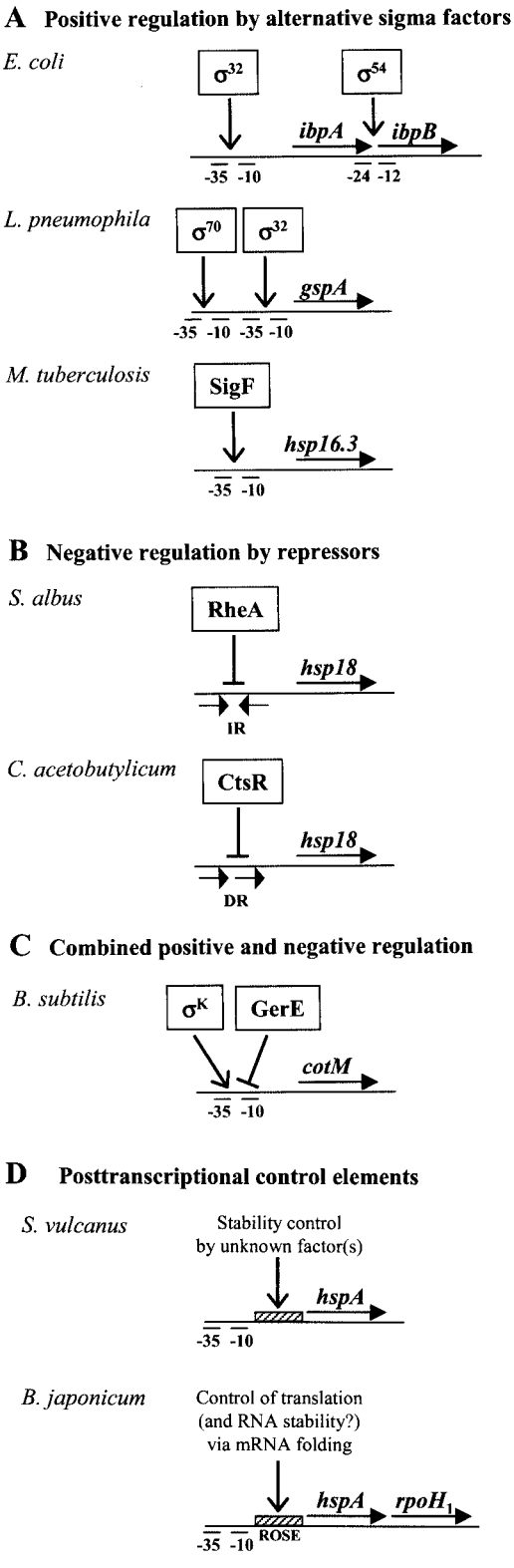

FIG. 3. Regulation of bacterial α-Hsp genes. A representative organisms for each regulatory strategy is listed. The regulated genes and corresponding regulators are indicated. Promoters are presented as −10 and −35 regions. Known repressor-binding sites occur as inverted repeats (IR) or directed repeats (DR). Details are presented in the text.

pressor is unable to interact with its target sequence (an inverted repeat) at elevated temperatures (274). The transition between the active and inactive forms is fully reversible. RheA thus acts as a cellular thermosensor, directly correlating heat shock gene expression to the ambient temperature.

hsp18 of *Clostridium acetobutylicum, hsp18* of *Leuconostoc oenos* (*Oenococcus oeni*), and *hsp16.4* of *Streptococcus thermophilus* are preceded by putative CtsR target sites (64). CtsR (for class three stress gene repressor) was initially identified in *B. subtilis* as negative regulator of the *clpC* operon (64, 163). It blocks transcription of target genes at normal temperatures through binding to a conserved heptanucleotide repeat (Fig. 3B). CtsR is specifically degraded by the ClpCP protease during stress. Since expression of the Clp components (and some additional regulatory factors) is under CtsR control, proteolysis of the repressor constitutes a built-in autoregulatory loop (65, 164). Putative *ctsR* homologs and potential CtsR-binding sites upstream of heat shock genes were documented in a number of gram-positive microbes (64). Temperature-regulated transcription from housekeeping promoters was demonstrated for the *C. acetobutylicum* and *O. oeni hsp18* genes (142, 267). However, experimental proof that CtsR mediates heat shock control of α-Hsp genes in these and other gram-positive bacteria remains to be generated.

Neither of the three α-crystallins of *B. subtilis* is temperature regulated. CotM is induced at late stages during sporulation and maintains the structural integrity of the outer spore coat (120). Both an alternative sigma factor (the late sporulation sigma factor σ^K) and a repressor protein (GerE) contribute to its proper temporal and spatial expression (Fig. 3C). Similar dual control of *ydfT* led to its alternative designation, *cotP* (255). Although expression of CotM and CotP is restricted to sporulation, they seem to be dispensable for this process.

A number of bacterial α-Hsp genes and operons are regulated by mechanisms that cannot be assigned to either transcriptional activation or repression. Long untranslated regions (5′UTRs) were found upstream of various rhizobial α-Hsp genes and upstream of *hspA* of *Synechococcus vulcanus* (223, 224, 235, 263) (Fig. 3D). Temperature-regulated transcription of all these genes was initiated at vegetative promoters. A novel regulatory mechanism controlling mRNA stability in a temperature-dependent fashion was inferred from the fact that the *hspA* transcript of *S. vulcanus* is more stable at 63 than 50°C. Unknown factors might be involved in this process (263). The 5′UTR of at least 15 small heat shock operons from *B. japonicum, Bradyrhizobium* sp. (*Parasponia*), *Rhizobium* sp. strain NGR234, and *Mesorhizobium loti* was designated ROSE, acknowledging its role in repression of heat shock gene expression (223, 235). Although a repressor-type mechanism was favored initially, there now is accumulating evidence that ROSE acts at the posttranscriptional level. RNA structure predictions suggest that the highly conserved 3′ region of all known ROSE sequences folds into a stem-loop structure that masks the ribosome-binding site (RBS) (234). A detailed mutagenesis study on a representative ROSE element demonstrated that the exchange of individual nucleotides potentially involved in base pairing relieves α-Hsp gene repression at low temperatures. The current model holds that mRNA folding at low temperatures has two consequences. It blocks access of ribosomes to the RBS and thereby impairs translation. The

presumed secondary structure probably also promotes RNase cleavage and rapid decay of ROSE-containing mRNAs, explaining the minute amounts of α-Hsp transcripts at low temperatures. According to this model, high temperatures would melt the hairpin structure, allowing ribosomes to access the RBS. Ribosome entry then would initiate translation and simultaneously protect ROSE-containing mRNA from degradation.

A noteworthy feature of several α-Hsp genes is their extrachromosomal location. Small plasmids carry the low-molecular-mass stress proteins of *Streptococcus thermophilus* (239, 298, 299). On plasmids pER16 (4.5 kb), pER35 (10 kb), pER36 (3.7 kb), and pER341 (2.8 kb) originating from different *S. thermophilus* strains, the only other open reading frame encodes a rolling-circle replication protein (298, 299). The 6.5-kb plasmid pCI65st from *S. thermophilus* NDI-6 codes for two almost identical α-Hsps (Hsp1 and Hsp2; an alignment is given in Fig. 2), a replication protein, a putative enolase, and HsdS, a putative type 1 restriction modification enzyme (239). These streptococcal replicons are readily lost at low temperatures but stably maintained at optimal growth temperatures of 42°C (239, 298). The plasmid stability suggests that the α-Hsps are beneficial to lactic acid bacteria during fermentation of dairy products.

Sequencing of rhizobial genomes revealed that some of their multiple α-Hsp genes are also located on extrachromosomal replicons. *M. loti* contains the megaplasmids pMLa (352 kb) and pMLb (208 kb). The latter bears the locus *mll9627*, coding for one of eight α-crystallins (143, 235). In *Sinorhizobium meliloti*, three of five α-Hsps are encoded on symbiotic megaplasmids. The locus SMa1118 lies on pSymA (1.4 Mb), and SMb21294 and SMb21295 are on pSymB (1.7 Mb) (89). The presence of several α-Hsps in bacteroids suggests that these proteins play a role in establishing a successful plant-microbe interaction (226). A plasmid-borne α-crystallin gene was also found in *Buchnera aphidicola*, the endosymbiont of aphids (334). Other genes harbored by this 8.5-kb plasmid are involved in leucine biosynthesis. A remarkably similar clustering of an *ibpB*-like gene with leucine biosynthetic genes was observed on the chromosome of *Azotobacter vinelandii* (195). Whether the close association of α-Hsp genes with amino acid metabolism genes is a coincidence or functionally relevant is unclear.

OLIGOMERIZATION OF α-HEAT SHOCK PROTEINS

Homo-Oligomeric Complexes

A hallmark of α-Hsps is their tendency to "socialize," or in other words, to assemble into large oligomeric complexes. There is ample evidence that oligomerization is a structural prerequisite for chaperone activity of the vast majority of α-Hsps. Neither naturally occurring multimerization-incompetent α-crystallins nor mutated variants that have lost the ability to form large complexes are efficient chaperones 160, 181, 182, 331; Studer et al., submitted). In many cases in which bacterial α-Hsps have been studied biochemically, they were reported to build complexes consisting of approximately 24 subunits (156, 204, 262, 310). Formal proof for such an organization stems from the crystal structure of the *M. jannaschii* Hsp16.5. A total of 24 monomers form a spherical complex with 14 open windows (155) (see Fig. 5). Electron microscopy (EM) images also revealed similar spherical complexes for human αB-crystallin, human Hsp27, native bovine α-crystallin, and yeast Hsp26 (107, 112).

Examples of both smaller and larger assemblies than 24-mers have been documented. *M. tuberculosis* Hsp16.3 forms a triangular structure comprising a nonamer composed of a trimer of trimers (44). Pea Hsp18.1 and Hsp17.7, two representatives of cytosolic plant α-Hsps, produce discretely sized complexes containing 12 identical subunits (174). Murine Hsp25 exists predominantly as a hexadecamer (72, 74). At the opposite extreme of the oligomeric scale are the proteins that form large and heterogenous assemblies. α-Crystallin from vertebrate eye lenses is normally isolated as an 800-kDa complex comprising 32 subunits. However, various other assemblies from 280 kDa to 10 MDa have been reported (101). The majority of *E. coli* IbpB is eluted from gel filtration columns as globular structures exceeding 2 MDa (282, 341). Electron micrographs reveal pronounced size heterogeneity from small roughly spherical complexes with a diameter of 15 nm (approximately 600 kDa) to 100–200-nm structures that interact over time to form loose aggregates of micrometer size (282).

Regardless of the subunit composition, all those diverse assemblies described above efficiently protect other proteins from aggregation, illustrating that a composition of 24 subunits is not a structural prerequisite for chaperone activity. The astounding diversity of α-Hsp assemblies also is the reason why the minimal oligomeric requirement for chaperone activity is not clear-cut. The monomeric Hsp12.6 from *Caenorhabditis elegans* is unable to prevent the aggregation of test substrates (181). Hsp12.2 and Hsp12.3, two other exceptionally small members of the α-crystallin family of *C. elegans*, assemble into tetramers that are devoid of any chaperone activity (160). In contrast, Hsp20 from rats tends to form dimers that act as poor chaperones (331). A truncated version of human αB-crystallin (αB57–157) that contains only the isolated α-crystallin domain builds dimers with significant chaperone activity (81). Unlike other α-Hsps, yeast Hsp26 requires temperature-assisted dissociation of a 24-mer into dimeric species in order to become an efficient chaperone (112). Tetramers of murine Hsp25 that occur as intermediates in the assembly of 16-mers have been shown to bind troponin T, a structurally labile marker protein for myocardial cell damage (73). This short list of observations already implies that the correlation between the oligomeric status and chaperone activity is different for individual α-Hsps.

Hetero-Oligomeric Complexes

The tendency of α-Hsps to generate higher-level structures poses the question whether oligomers always contain identical subunits or whether mixed oligomers can be formed (provided that different α-Hsps occur in the same cell or cellular compartment). Native α-crystallin isolated from the eye lens is composed of the closely related subunits αA- and αB-crystallins in a 3:1 stoichiometry (129). Under in vitro conditions, however, any ratio can be formed (333). Several reports demonstrate that αA- and αB-crystallins are able to interact with other members of the α-crystallin family. αB-crystallin copurifies with human Hsp28 or with rat Hsp27 (146, 367). On mixing

of purified components in one study, heteropolymers from any combination of bovine αA-crystallin, αB-crystallin, and mouse Hsp25 could be detected (202). An interaction between Hsp27 and αB-crystallin was demonstrated by the yeast two-hybrid system (27, 190). Finally, fluorescence-labeled αA-crystallin readily exchanged with αB-crystallin and with Hsp27 (31). All these findings strongly suggest that hetero-oligomerization is a common phenomenon in mammalian cells.

The formation of mixed α-Hsp complexes in plants and bacteria appears to be more restricted than in mammals. Most of the multiple α-Hsps that are being produced in plants are prevented from interacting with each other because they are housed in different cellular compartments (349). However, three distinct classes occur in the cytoplasm. The recombinant pea proteins Hsp18.1 and Hsp17.7 (representative class I and class II members, respectively) were analyzed for complex formation in vitro (119). Both proteins strictly assembled into homo-oligomeric complexes and did not form hetero-oligomers. EM revealed differences in the shapes of Hsp18.1 and Hsp17.7 complexes, which might prevent the exchange of subunits between α-Hsps from different classes (174). Isolated wheat Hsp16.9 and pea Hsp18.1, two class I proteins, readily exchanged subunits (338a). The finding that isolated native class I oligomers from soybean, rice, mung bean, and pea consist of at least 8 to 15 different isoforms also suggests that α-Hsps of the same class produce hetero-oligomeric complexes in vivo (141).

Separate formation of dodecameric complexes was also described for class I and class II proteins from tobacco protoplasts (157). Interestingly, class I- and class II-specific homo-oligomers coassembled into heat shock granules (HSGs), large cytosolic complexes that appear under stress conditions and disassemble during recovery (237). Their inner core is probably composed of α-Hsps, whereas Hsp70 and Hsp40 seem to be associated with the surface of the granules (296). Denatured proteins, mRNA, RNA-binding proteins, and heat shock transcription factors might also be enclosed in HSGs (149, 237, 268). The integration of α-Hsps into HSGs is not the result of non-specific aggregation but an ordered assembly process (157). Only class II complexes can jointly build the core of a HSG, whereas class I proteins cannot. Remarkably, preexisting class II supercomplexes can then recruit class I homo-oligomers. This assembly process is highly specific because plant α-Hsps from other classes are not incorporated into HSGs.

Copurification studies with bacterial α-Hsps provided direct evidence for complex formation between two members of the same class (310). Isolated hetero-oligomers displayed chaperone activity indistinguishable from either homo-oligomeric complex. Mixed oligomers were also obtained with class A proteins from different organisms, e.g., *E. coli* IbpB and *B. japonicum* HspB. However, they were never observed between members of two different classes, e.g., *E. coli* IbpB and *B. japonicum* HspC or *B. japonicum* HspB and HspC, suggesting a structural barrier for oligomerization of distantly related α-crystallins.

Is the formation of mixed oligomers the result of a fortuitous interaction of structurally related proteins, or might it be of functional significance? In principle, the multimerization of similar but not identical subunits offers the opportunity to take advantage of slightly different properties of the individual particles. Most likely, the differences between certain compositions are subtle and are detectable only by rigorous biochemical investigation. Advances in this direction have centered on the lenticular α-crystallins. Recombinant αB-crystallin was shown to be a better chaperone than αA-crystallin, in particular at physiological temperatures (57, 311). Optimal chaperone activity probably is sacrificed for stability reasons in the naturally occurring 3:1 stoichiometry of αA- and αB-crystallins. The mixed complex has greater thermal stability and is more compact than is either homo-oligomer (312). A stable and long-lived structure might be the ideal solution in the eye lens since there is no protein turnover in this tissue.

Apart from structural considerations, it is conceivable that variations in the subunit composition might alter the substrate specificity of α-crystallins. Little is known about substrate selection of these chaperones. If different α-crystallins were to recognize specific target sites in unfolded proteins, the coassembly of different subunits should influence the binding properties. It is plausible that the rearrangement of α-crystallin complexes provides a means of tailoring the quatenary structure and functional properties of α-crystallins according to the actual needs.

Plasticity of Oligomer Formation

The formation of heteromeric structures suggests that α-Hsp assemblies are dynamic and are shaped by the frequent exchange of subunits. In fact, EM examination of various family members revealed highly variable quaternary structures (74, 107, 108, 112, 186, 282). Processed cryo-EM images uncovered a high degree of plasticity in the quaternary structure. Human αB-crystallin particles ranged in diameter from 80 to 180 Å, native bovine lens α-crystallin (3:1 molar ratio of αA- and αB-crystallins) ranged from 120 to 200 Å, and human Hsp27 ranged from 90 to 220 Å (107, 108). Unlike other family members, *Methanococcus jannaschii* Hsp16.5 was monodisperse and formed a rigid structure of rather homogenous size between 110 and 130 Å (107, 156). Apparently, this precisely defined particle was a proper choice for crystallization and structure determination (155). Despite a wide range in particle diameter and subunit composition, most α-Hsps imaged so far seem to fashion spherical assemblies with a central cavity, similar to the Hsp16.5 complex. It is as if football-like structures can be achieved with different numbers of subunits.

In an ordered assembly pathway, smaller particles serve as building blocks for multimeric complexes. Three trimers, for instance, produce the triangular nine-subunit complex of *Mycobacteriumtuberculosis* Hsp16.3 (44) (Fig. 4A). Dimers have been postulated as building blocks of the dodecameric Hsp16.5 particle of *Methanococcus jannaschii* (155) (Fig. 4B). Intermediates of the assembly pathway were not revealed by size exclusion chromatography since Hsp16.5 eluted as a single sharp peak representing a 24mer (156). In other instances, α-Hsp complexes are in a highly dynamic equilibrium with smaller and larger particles. The hexadecameric murine Hsp25 is in a concentration-dependent equilibrium with tetramers and dimers, indicating that both particles occur during the assembly pathway (74) (Fig. 4C). Elevated temperatures induced the cooperative association of hexadecamers to large

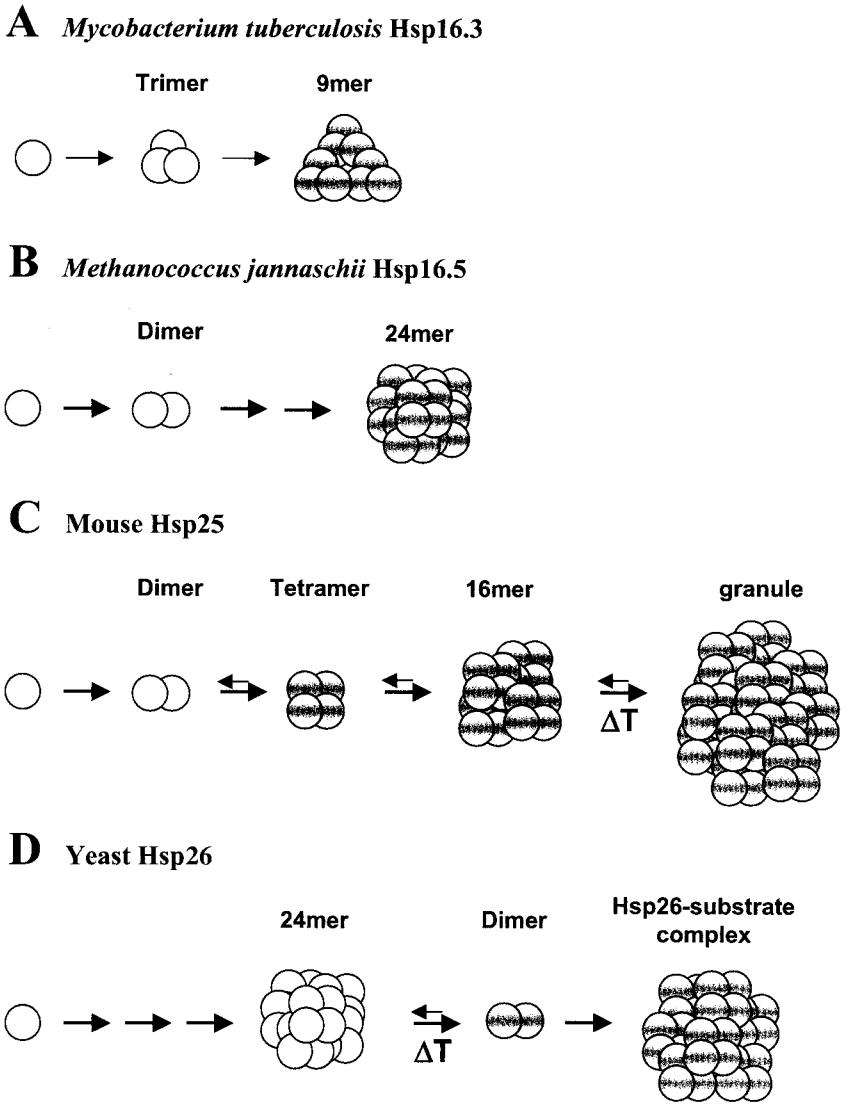

FIG. 4. Postulated assembly pathways of α-Hsp complexes. Particles competent for binding unfolded proteins are shaded in gray. ΔT indicates a temperature upshift. For further details, see the text.

granules, which exist in slow equilibrium with the smaller particles. Quite remarkably, tetramers, hexadecamers, and granules of Hsp25 acted as chaperones in suppressing protein aggregation. A completely different pathway was postulated for Hsp26 from *Saccharomyces cerevisiae* (112). This protein is an efficient chaperone only at elevated temperatures. Heat shock temperatures trigger the dissociation of a 24-mer of Hsp26. The resulting dimers recognize and bind substrate proteins before they reassemble into large chaperone-substrate complexes clearly exceeding the original complexes (Fig. 4D). It was concluded that yeast Hsp26 is a temperature-regulated chaperone whose activation is achieved by dissociation of a 24-mer that can be regarded as inactive storage form of the chaperone.

Comparative size exclusion chromatography of Hsp26 and bovine α-crystallin showed that the thermoresponsiveness found for Hsp26 is not a mechanism common to all α-Hsps. The α-crystallin complex did not disassemble during heat treatment (112). It is evident that the α-crystallin-type proteins that are induced in response to stimuli other than heat stress must act differently from Hsp26. Many α-Hsps function as efficient chaperones not only on thermally but also on chemically denatured substrates at low temperatures in vitro (e.g., on dithiothreitol [DTT]-treated insulin) (1, 188, 253). While temperature-mediated dissociation of α-Hsps might not be a universal phenomenon, more subtle temperature effects on the structural properties, chaperone activity, and subunit exchange of various family members are common. Of all parameters examined (temperature, ionic strength, pH, and Ca^{2+} concentration), temperature had the most pronounced effect on subunit exchange (30). The exchange rate of αA-crystallin subunits as monitored by fluorescence resonance energy transfer was facilitated by a temperature shift from 37 to 42°C. Several other reports indicate that high temperatures induce structural transitions in α-Hsp complexes (57, 250, 253, 282, 335, 338). A temperature increase triggers the exposure of hydrophobic res-

idues, a structural reorganization that is thought to promote both subunit exchange and binding of unfolded proteins. Hetero-oligomers are probably more susceptible to temperature-driven conformational changes than are homo-oligomers since it was shown that complexes between Hsp27 and αB-crystallin were disrupted at 44°C (367). Complete reconstitution of mixed complexes occurred during recovery at 37°C.

Various posttranslational modifications, including phosphorylation, deamidation, acetylation, and glycosylation, have been detected for mammalian α-Hsps (11, 62, 71, 101, 193). Phosphorylation on one or more serine residues occurs in different tissues and in response to a wide array of environmental stimuli such as heat, arsenite, and growth factors. In the context of oligomerization, it is notable that phosphorylation often is associated with changes in the oligomeric structure of α-Hsp complexes. The correlation between phosphorylation, oligomer formation, and chaperone activity differs between individual α-Hsps (71, 193). However, in most cases, phosphorylation is associated with a reduction of the multimeric size. Hsp27, Hsp20, and αB-crystallin complexes are downsized by phosphorylation (17, 136, 145, 168, 259). Phosphorylation-induced dissociation of Hsp27 and αB-crystallin concomitantly reduce chaperone activity (136, 259). Before phosphorylation of maize mitochondrial Hsp22 was detected recently (191a), phosphorylation of α-Hsps was thought be an animal-specific process (314, 349).

STRUCTURE OF α-HEAT SHOCK PROTEINS

Although lenticular α-crystallins have been the subject of intense investigation for many years, high-resolution structures are still unavailable. The dynamic quatenary structure of these proteins and numerous posttranslational modifications are thought to be the reason for their intrinsic resistance to crystallization trials (101). Numerous and in part controversial structural models have been proposed on the basis of known physicochemical properties of α-crystallins. Several competing three-layer models with variable numbers of subunits, a micelle-like structure, a "pitted-flexiball," a "bean with tentacles," and a GroEL-type complex are among the hypothetical models (42, 78, 101, 295, 336).

Microscopic examination of negatively stained samples revealed roughly spherical complexes for many members of the α-crystallin family, e.g., for mouse Hsp25, yeast Hsp26, human Hsp27, pea Hsp18.1 and Hsp17.7, *Bradyrhizobium japonicum* HspB and HspC, and *Methanococcus jannaschii* Hsp16.5 72, 74, 112, 156, 174, 175, 259; Studer et al., submitted). As mentioned above, cryo-EM images showed nonameric triangles of *Mycobacterium tuberculosis* Hsp16.3, a structural organization presently unique among α-Hsps (44). Three-dimensional reconstruction of cryo-EM images of human αB-crystallin, native bovine α-crystallin, human Hsp 27, and *M. jannaschii* Hsp16.5 visualized spherical complexes with an inner cavity (107, 108). The overall picture of the latter protein thus obtained closely matches its known crystal structure (155).

Whereas the first 32 residues of *M. jannaschii* Hsp16.5 are highly disordered in the crystal, the structure from residue 33 onwards, including the entire α-crystallin domain (residues 46 to 135) and the C-terminal extension (136 to 147), could be determined at 2.9 Å resolution (155). Twenty-four subunits are arranged with octahedral symmetry in a spherical complex containing eight triangular and six square openings (Fig. 5A). The outer and inner diameters of the hollow sphere are around 120 and 65 Å, respectively. Each monomer consists of 10 β-strands and 2 short helices (Fig. 2). The core of Hsp16.5 adopts an immunglobulin-like fold consisting of two β-sheets that are packed as parallel layers. β1, β7, β5, and β4 form one β-sheet, and β2, β3, β9, and β8, together with β6 of a neighboring subunit, form the other β-sheet (Fig. 5B). The donated strand is located in the center of the α-crystallin domain (Fig. 2). Each subunit in the complex makes extensive contacts with other subunits via hydrogen bonds as well as hydrophobic and ionic interactions. The short C-terminal extension is oriented toward the outside of the shell and interacts with β4 and β8 of a neighboring subunit. Although the size of the windows on the surface of the sphere would allow the passage principally of small molecules and unfolded polypeptide chains, it is unlikely that client proteins of Hsp16.5 are housed in the central cavity. Several lines of evidence, including them the presence of internal density in the Hsp16.5 shell in cryo-EM images, strongly indicate that the interior of Hsp16.5 and other α-Hsp complexes is filled with the disordered N-terminal region (107, 155, 182).

What does the Hsp16.5 structure tell us about the possible structure of other members of the α-crystallin family? The high-resolution structure of complexes that consist of 9, 12, 16, 32, or another number of subunits will certainly deviate from the 24-mer of Hsp16.5. Nevertheless, principal features of the Hsp16.5 structure might pertain to other α-Hsps, even if they do not form spherical assemblies. This assumption is nicely supported by the recently solved crystal structure of wheat Hsp16.9 (338a). The structures of monomeric and dimeric subparticles in the Hsp16.5 and Hsp16.9 complexes are very similar. Nevertheless, the overall quaternary structures are different. Wheat Hsp16.9 assembles not into spherical complexes but into a dodecameric double disk. Circular dichroism studies and secondary-structure predictions indicate that other α-Hsps also consist predominantly of β-sheets (32, 181, 202, 215, 282). It is important to note, however, that many residues involved in subunit contacts of *M. jannaschii* Hsp16.5 are not conserved in other family members (155). Most strikingly, the region around β6, which is intimately involved in monomer-monomer interactions, is extremely variable among α-Hsps or even absent, depending on the gap positioning in a given sequence alignment (compare Fig. 2 and the alignment in reference 155). Despite the low sequence identity, a β-sandwich-like organization similar to that in the *M. jannaschii* protein was also demonstrated in the α-crystallin domains of αA-crystallin, Hsp27, and *M. tuberculosis* Hsp16.3 by site-directed spin labeling (22, 162). The β-strands of *M. jannaschii* Hsp16.5 and of αA-crystallin are illustrated at the top and bottom of Fig. 2, respectively. Such comparative analyses show that the immunoglobulin-like fold of α-Hsp monomers is highly conserved. The divergence in the primary amino acid sequence, in particular in the flanking N- and C-terminal regions, most probably is responsible for the amazing differences in size, symmetry, and dynamics of the actual oligomeric structures.

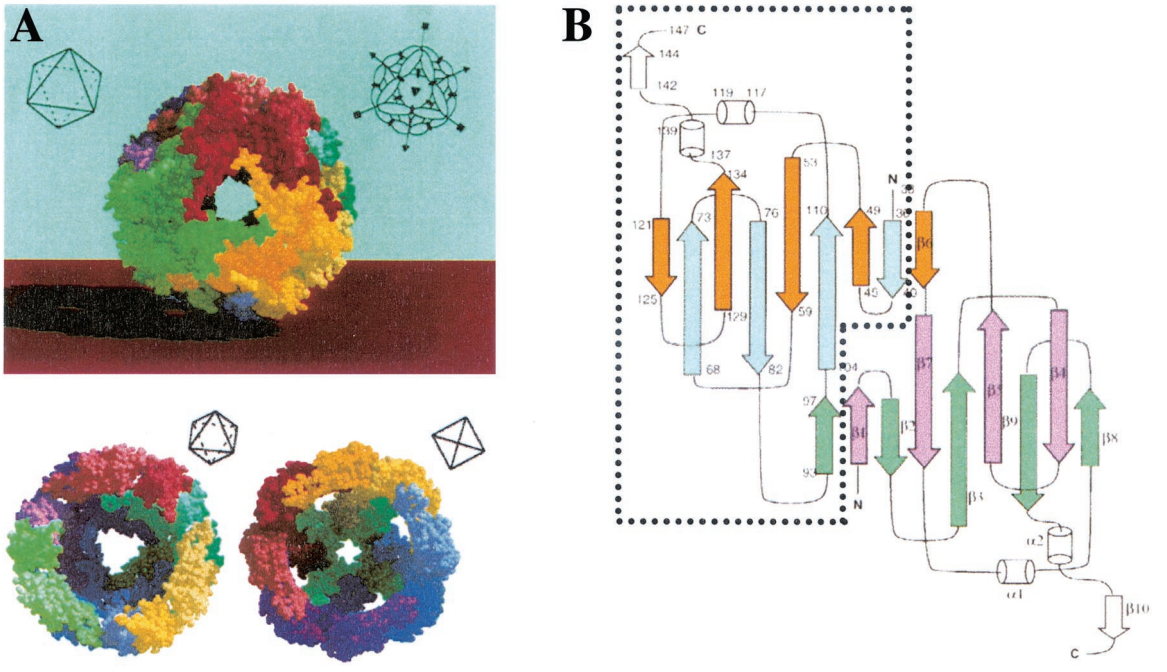

FIG. 5. Overall structure and subunit interactions of *Methanococcus jannaschii* Hsp16.5. (A) In the space-filling model of the hollow sphere, each tetramer is represented in one color with different shadings. At the bottom, the interior of the sphere is viewed along the threefold axis (left) and the fourfold axis (right). The front one-third of each sphere is cut off. (B) Topology of the secondary structure of a Hsp16.5 dimer. The first and last residue numbers for each secondary-structure element are indicated in the left monomer, which is indicated by a dotted frame. The structural elements are labeled in the right monomer. (Reprinted from reference 155 with permission of the publisher.)

FUNCTIONAL REGIONS OF α-HEAT SHOCK PROTEINS

Regions Responsible for Oligomerization

α-Crystallin domain. The structural data outlined above leave little doubt that the α-crystallin domain is engaged in extensive subunit-subunit contacts. Congruent is the finding that a genetically engineered human αB-crystallin comprising only the α-crystallin domain forms a dimer (81). Yeast two-hybrid system analyses provide genetic evidence for interactions in the α-crystallin domain (27, 157, 168, 190, 359). The conserved motif A-x-x-x-n-G-v-L (Fig. 2) toward the C-terminal end of the α-crystallin domain was identified as an important determinant for the interaction of yeast Hsp42p (359). Efforts to identify individual amino acid residues involved in subunit interaction and/or chaperone activity by point mutagenesis had limited success owing to the astounding robustness of α-crystallins to limited perturbations (63, 128, 162, 218, 295). Although some point mutations resulted in significantly enlarged assemblies, chaperone efficiency was barely affected (128, 295).

Interestingly, two disease-related point mutations of a highly conserved arginine at an equivalent position in αA-crystallin (R116C) and αB-crystallin (R120G) introduce functionally relevant structural alterations (32, 50, 166, 244, 288). The αA-crystallin mutation occurs in patients suffering from hereditary cataracts. The αB-crystallin mutation causes desmin-related myopathy, a neuromuscular disease characterized by the accumulation of cytoplasmic inclusion bodies containing the defective αB-crystallin with the filament protein desmin. The mutated arginine of αA-and αB-crystallin is located in β-strand C3 (Fig. 2). It corresponds to an arginine in β7 of *Methanococcus jannaschii* Hsp16.5 and is moderately conserved throughout other α-Hsps. The arginine exchange derivatives have an oligomeric size twice to four times as large as the authentic proteins. Cryo-EM images of R120G-αB-crystallin revealed an irregular shape that lacks a well-defined central cavity (32). The conformational changes in R116C-αA-crystallin and R120G-αB-crystallin coincide with significantly reduced chaperone activity (32, 50, 166, 244, 288). The equivalent mutation (R100C) in *Mycobacterium tuberculosis* Hsp16.3 resulted in a protein that had only 40% of the wild-type chaperone activity (22). In contrast to the human α-crystallin proteins, the arginine exchange in Hsp16.3 resulted in a reduced complex size. Cysteine substitutions of S91, E92, F93, and Y95 also led to dissociation of the nonameric complex into smaller oligomers. This indicates that the region including and preceding the R100 residue serves as interface for the assembly of the trimeric building blocks of Hsp16.3. All other single-cysteine mutants between position 91 and 105 had no impact on oligomerization and chaperone activity.

N-terminal region. A second important conclusion can be drawn from the dimeric state of the isolated α-crystallin domain mentioned above (81). Obviously, the central domain alone is not sufficient for the formation of higher-order structures. To assess the role of the N-terminal region and the C-terminal extension in α-Hsp assembly, the four Hsp12 proteins of *Caenorhabditis elegans* have been investigated. They are the smallest naturally occurring representatives of the α-crystallin family and are reduced to the α-crystallin domain preceded by an exceptionally short N-terminal region of 25 or

26 residues (160, 181). Although Hsp12.6 eluted as a dimer during size exclusion chromatography, sedimentation velocity experiments revealed a monomeric protein (181). Evidently, this result does not support any contribution of the α-crystallin domain to dimerization and subsequent oligomer assembly. In contrast, Hsp12.2 and Hsp12.3, two other miniature α-crystallin-type proteins of *C. elegans*, readily formed complexes up to tetramers (160). Neither of the Hsp12 proteins had detectable chaperone activity in vitro (160, 181). Their expression is developmentally regulated and unaltered by stress conditions. Since it is assumed that these proteins have specialized functions independent of oligomerization and chaperone activity, they may not be the best choice for drawing general conclusions on requirements for complex formation. Hsp16-2, yet another α-crystallin member of *C. elegans*, served as alternative model protein for studies on multimerization (182). The α-crystallin domain of this heat-inducible protein is flanked by a typical N-terminal region (41 residues) and a C-terminal extension (22 residues). Furthermore, purified Hsp16-2 is a classical chaperone that prevents thermally and chemically induced protein aggregation. The recombinant protein occurred in two subpopulations consisting of approximately 14 and 24 subunits. N-terminally truncated versions lacking 15, 32, or 44 amino acids failed to form native-like complexes, but trimeric and tetrameric species were discernible after cross-linking (182). Neither of the truncated Hsp16-2 proteins had chaperone activity. These studies clearly established a critical role of the N-terminal region in the assembly of low-molecular-mass species into functional high-molecular-mass complexes.

Since there is considerable deviation in the N-terminus of α-Hsps (Fig. 2), it is difficult to define the exact upper and lower limits of sequence length and the amino acid composition required for proper assembly of multimeric complexes. Human αA-crystallin devoid of the first 20 amino acids retained its high-molecular-mass structure, whereas a truncation of 56 residues reduced the complex to trimers or tetramers (31). The examination of truncated versions of *Bradyrhizobium japonicum* HspH (class A) and HspF (class B) also demonstrated that clipping off a few residues from the N terminus was tolerated without obvious defects in oligomerization and chaperone function whereas the removal of more than 5 to 10 amino acids was deleterious to both activities (Studer et al., submitted).

Interestingly, the N termini of certain α-Hsps can be remarkably insensitive to sequence extensions. Adding a tail of 4 -kDa that contains a hexahistidine tag to the N terminus of *C. elegans* Hsp16-2 did not disturb its multimerization capacity (182). The observation that the tagged protein bound to nickel-chelate affinity resin only in the presence of urea suggested that the N terminus is not exposed but sequestered in the interior of the complex. When maltose-binding protein (42 kDa) was added to the N terminus of αB-crystallin, the fusion protein still associated into high-molecular-mass oligomers that displayed full chaperone activity (215). This intriguing feature demonstrates that there is sufficient space in the cavity of the mammalian complex to accommodate N termini of various lengths. In contrast, the N termini of plant α-Hsps seem more sensitive to sequence additions. Fusion of glutathione *S*-transferase (29 kDa) to tobacco Hsp18 blocked oligomer formation at the dimeric stage (297).

C-terminal extension. Whereas the importance of a largely intact N-terminal domain is well documented, the exact contribution of the C-terminal extension to complex formation is debated. Much like the N-terminal region, the length and sequence of the individual C-terminal ends vary considerably (Fig. 2). Point mutations or amino acid insertions in the last 4 residues of bovine αA-crystallin had little effect on the molecular mass of the complex (294). With one exception, chaperone activity was not disturbed when a hydrophobic tryptophan residue was introduced. The complete removal of the C-terminal extension from *C. elegans* Hsp16-2 had little impact on oligomerization and chaperone ability (182). The complexes obtained were slightly larger than wild-type complexes and readily precipitated after freeze-thawing. This was taken as evidence that one function of the C-terminal tail is to solubilize the α-Hsp complex. Mouse Hsp25 without its C-terminal extension also showed a normal oligomeric mass and suppressed thermal aggregation of CS (186). Surprisingly, the protein was unable to prevent DTT-induced precipitation of α-lactalbumin, implying that its flexible C-terminal tail, although not involved in oligomerization, is required for chaperone activity with certain substrates.

The oligomeric status of several other α-Hsps is considerably more sensitive to alterations in their C-terminal extensions. In line with the contention that the carboxyl end acts as solubilizing agent of α-Hsp complexes is the intrinsic insolubility of human αA-crystallin with the last 17 amino acids deleted (8). Precipitation goes along with markedly reduced chaperone activity. Comparable results were reported for *Xenopus* Hsp30C. Deletion of 25 amino acids from the carboxyl end resulted in reduced solubility and impaired chaperone function (82).

Reports on plant and bacterial α-Hsps demonstrate that the correct assembly into high-molecular-mass complexes depends on their carboxy termini. C-terminally shortened pea Hsp17.7 variants lacking 3, 5, or 10 amino acids were defective in the formation of full-size oligomers. In contrast to wild-type-like 250-kDa complexes, they formed assemblies of approximately 130, 100, and 67 kDa, respectively (157). Only small complexes, probably dimers, were detected when C-terminally truncated *B. japonicum* α-Hsps lacked the conserved I-X-I motif (sequences are given in Fig. 2). Even the single replacement of either isoleucine by alanine completely impaired multimerization and consequently chaperone activity (Studer et al., submitted). This finding assigns an important structural role to this motif that is present in many, but not all, bacterial α-Hsps. The comparatively short extension of *M. jannaschii* Hsp16.5 carries both isoleucines followed by just one other residue (glutamic acid; Fig. 2). The crystal structure shows that the tail reaches out of the sphere and that both isoleucines are involved in hydrophobic interactions with neighboring subunits (155). The C-terminal tail of wheat Hsp16.9 plays a similar critical role in stabilization of the oligomer (338a). Mutational studies of Hsp16.5 or Hsp16.9 have not been carried out. However, it is reasonable to assume that point mutations in the carboxyl end would destabilize and dissociate the complexes.

How can the discrepancies between reports concerning the role of the C-terminal extension be reconciled? Obviously, oligomerization is a very complicated process in which the actual requirements for individual α-Hsps might well be dif-

ferent. At least some bacterial α-Hsps are known to form highly ordered structures, in contrast to their polydisperse mammalian counterparts (44, 74, 107, 155). The C-terminal ends of *M. jannaschii* Hsp16.5 and of wheat Hsp16.9 are rigid and amenable to crystal structure determination (155, 338), whereas the tail of mammalian α-crystallin-type proteins reportedly is highly flexible (42). It is therefore likely that bacterial (and plant?) α-Hsps are more susceptible to mutational alterations. The exact positioning of all three functional regions might be more critical for the correct assembly of α-Hsps from these organisms. In this respect, it is interesting that more than 150 single-cysteine mutations in human αA-crystallin and 20 mutations in Hsp27 did not result in complex dissociation (22). On the other hand, 6 of 15 mutations in *M. tuberculosis* Hsp16.3 severely interfered with the assembly of native-like complexes. This comprehensive data set implies that only assemblies that are very dynamic can accommodate various point mutations or truncations without deleterious effects. It might therefore be worthwhile to carry out systematic site-directed mutagenesis studies on bacterial and plant α-Hsps.

Substrate-Binding Sites

The previous sections repeatedly emphasized the stringent coupling of complex formation and chaperone activity. Only certain oligomeric states, which vary among α-Hsp species, seem to orient interactive surfaces properly to promote substrate binding. Since the exposure of hydrophobic regions is characteristic of (un)folding intermediates, it has been surmised that hydrophobicity is an important parameter for chaperone-substrate interaction. In keeping with this conjecture is the finding that the chaperone efficiency of α-crystallins correlates with their degree of hydrophobicity. Far-UV circular dichroism and labeling with the fluorescent dye 1-anilino-8-naphthalene sulfonate (ANS) revealed that αB-crystallin has a greater content of α-helices and is more hydrophobic than αA-crystallin (57, 311). These structural features coincide with higher chaperone activity, indicating that hydrophobic interactions play an important role in substrate interaction. Furthermore, it has been reported that the temperature-driven exposure of hydrophobic surfaces in α-crystallin is accompanied by enhanced chaperone activity (250, 253). A temperature increase within the physiological range between 22 and 45°C triggered the exposure of buried hydrophobic regions in pea Hsp18.1, as was shown by the progressive increase of 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid (bis-ANS) incorporation (175). In the same temperature frame, *E. coli* IbpB undergoes similar conformational changes that alter surface hydrophobicity (282).

A number of studies made use of ANS, bis-ANS, or related small aromatic molecules to photolabel and subsequently localize potential substrate-binding sites in α-crystallin-type proteins. It is presumed that the regions interacting with these substances and with substrate proteins are identical for several reasons. First, preincubation of denatured alcohol dehydrogenase (ADH) with bovine α-crystallin or malate dehydrogenase (MDH) with pea Hsp18.1 blocked photoincorporation of the label (175, 280). Conversely, the binding of bis-ANS reduced the chaperone activity of α-crystallins (279, 293). Finally, regions identified by bis-ANS labeling overlapped with sites that cross-linked to substrate proteins (278, 281). Taken together, these data indicate that fluorescent probes and substrate proteins have common binding sites implicated in the chaperone activity of α-Hsps.

The outcome of photolabeling experiments with different α-Hsps varies. Incorporation of bis-ANS into rat αB-crystallin was restricted to the N-terminal domain (293). The labeled chaperone had considerably lower activity in the insulin assay than did untreated αB-crystallin, suggesting that a substrate-binding domain had been targeted by the label. Incorporation of fluorescent dyes into the amino-terminal region was also observed in bovine αA-crystallin and in pea Hsp18.1 (175, 280). The initial residues at the N terminus of Hsp18.1 and a short patch proximal to residue 50 of bovine αA-crystallin (QSLFR; highlighted in yellow in the human sequence in Fig. 2) were photolabeled. The exchange of two conserved phenylalanines (F24 and F27) near the N terminus of αB-crystallin led to chaperone deficiency, although the oligomeric properties were not grossly changed (247). This result can be taken as further evidence that the N-terminal region of α-crystallin contributes to substrate binding. It should be noted, however, that this result is disputed and that other studies showed no discernible chaperone defect of the phenylalanine exchange derivatives (63, 128).

In contrast to the work on rat αB-crystallin, additional bis-ANS labeling was demonstrated in the α-crystallin domain of bovine αA- and αB-crystallins and of pea Hsp18.1 (175, 280). The corresponding peptides in the human α-crystallin proteins and the sequence in *Methanococcus jannaschii* Hsp16.5 equivalent to the region identified in Hsp18.1 are shown in yellow or green in Fig. 2. Interestingly, the deduced substrate-binding site of Hsp16.5 maps in a loop that links β3 and β4. The crystal structure indicates that this loop is surface exposed and therefore well suited for protein-protein interactions (155). Several residues in this loop are involved in intersubunit contacts (79, 155). Likewise, in wheat Hsp16.9, several oligomerization interfaces coincide with putative substrate-binding sites, which led to the proposal that heat-induced chaperone activity is triggered by dissociation of the α-Hsp complex (338a). An intriguing feature of the presumed substrate-binding regions is the presence of several charged residues (Fig. 2). It has been proposed that such residues serve to generate optimal spacing of nonpolar residues and aid in hydration of the region (175). It is also conceivable that the chaperone and substrate interact via charged residues. Ionic pairing in addition to hydrophobic interactions might be useful for strengthening the stability of the chaperone-substrate complex, in particular when long-term storage of unfolded polypeptides is desired, as it is in the eye lens. Aside from their potential role in substrate binding, negative charges in the α-crystallin domain seem to be important to establish electrostatic interactions that stabilize the chaperone complex (27).

Cross-linking studies with denatured ADH or mellitin, a 2.8-kDa hydrophobic polypeptide, to bovine αA- and αB-crystallins disclosed similar sites to those defined by bis-ANS labeling (278, 281) (Fig. 2). Notably, the regions of αB-crystallin that generated a cross-link to ADH or mellitin were in close proximity but not identical. Two ADH-binding sites were located between residues 57 and 69 and residues 93 and 107, whereas the single mellitin-binding site encompasses amino

acids 75 to 82. Whether different client proteins are indeed sequestered to different chaperone sites in α-Hsps remains to be established. The fact that pre-incubation of αB-crystallin with insulin outcompeted chaperone activity against α-lactalbumin suggests that the binding sites are at least partially overlapping (305). Residues highlighted in green in Fig. 2 were identified by both the bis-ANS and cross-linking techniques (278, 280, 281). They cluster in an area corresponding to β3 to β5 in *M. jannaschii* Hsp16.5. Most strikingly, a synthetic 19-amino-acid peptide (KFVIFLDVKHFSPEDLTVK; residues 70 to 88 in αA-crystallin) prevented thermal aggregation of ADH, although with eightfold lower efficiency than did full-length αA-crystallin (281). This peptide also bound bis-ANS, strengthening the claim that bis-ANS-labeling sites and substrate-binding regions can be identical. The fact that Phe71, the second residue of that peptide, is essential for chaperone activity of rat αA-crystallin, lends additional support to the relevance of this region (266a). Although the participation of other regions cannot be excluded, it clearly emerges from these studies that the N terminus of the α-crystallin domain constitutes an important functional element for chaperone activity.

FUNCTION OF α-HEAT SHOCK PROTEINS IN PROTEIN QUALITY CONTROL

Numerous light-scattering studies demonstrate that α-Hsps act as molecular chaperones in the sense that they interact with target proteins and prevent their precipitation (44, 72, 74, 112, 127, 138, 156, 174, 175, 182, 202, 215, 262, 282, 310). α-Hsps are able to capture thermally or chemically denatured model substrates. Binding to the chaperone does not protect a substrate from its inactivation. The inactivation kinetics of several test enzymes was similar regardless of whether α-Hsps were present (44, 72, 174, 262). Moreover, MDH complexed with Hsp18.1 is hypersensitive to proteinase K treatment whereas MDH alone is resistant (175). Both findings indicate that α-Hsps recognize and sequester unfolded structures rather than native proteins. Bound substrates are thought to be in a flexible, molten-globule-like state in which unfolding is incomplete and secondary structure is preserved (55, 56, 187, 249). The fluorescence characteristics of tryptophan residues in α-crystallin-bound substrates were closer to those of native proteins than to those of fully unfolded ones (56). This is consistent with the idea that α-Hsps specifically recognize labile unfolding intermediates that occur early in the denaturation pathway.

In contrast to the GroEL complex that accommodates one or two substrate molecules per 14 subunits (111), α-Hsp assemblies have a much higher binding capacity. Many studies document that a molar (monomeric) chaperone to substrate ratio of 1:1 suffices to almost completely abolish light scattering (44, 72, 112, 127, 138, 310). Substrate-binding efficiency can vary depending on the temperature and model protein investigated. A 40:1 molar ratio of *Methanococcus jannaschii* Hsp16.5 to CS was necessary to prevent light scattering at 40°C (156). However, at 80°C, a temperature close to the growth optimum of 85°C for this organism, it protected single-chain monellin at a 1:1 molar ratio. Size exclusion chromatography demonstrated that pea Hsp18.1 could be saturated with 12 MDH molecules per dodecamer whereas it bound only an average of 0.25 CS subunit per monomer (175). The relative binding capacity of α-crystallin for DTT-sensitive proteins ranged from 2:1 (α-crystallin to insulin or α-crystallin to α-lactalbumin), over 8:1 (α-crystallin to bovine serum albumin) to 10:1 (α-crystallin to ovotransferrin) (188). The molecular masses of these four model substrates are 5.7, 14, 66, and 78 kDa, respectively. The clear correlation between low molecular mass of a substrate and high binding capacity of the chaperone implies that steric factors dictate the number of molecules that can be positioned on the surface of the α-Hsp complex. The finding that the rate of subunit exchange of αA-crystallin was markedly reduced on interaction with large target proteins indicates also that extended polypeptides interact simultaneously with multiple subunits of the chaperone (30).

Once formed, the chaperone-substrate complex is very stable. For instance, *E. coli* IbpA and IbpB bound tightly to inclusion bodies formed during heterologous protein production, a feature that led to their designation as "inclusion body-associated proteins" (6). Both proteins could also be recovered from endogenous *E. coli* proteins that had aggregated during heat treatment (170). *In vitro*, CS remained stably associated with Hsp25 for several hours (72). Likewise, complexes formed between Hsp18.1 and diverse substrates were stable for weeks at room temperature and were not disrupted by addition of ATP or salt or by storage at 4°C (175). Irreversible binding of unfolded proteins might be the only activity of lenticular α-crystallins, since there is no protein turnover in the eye lens. However, it is important to emphasize that polypeptides recruited by α-Hsps are not necessarily stuck in a dead-end conformation. CS could be released from Hsp25 and reactivated by the addition of oxaloacetate, a substrate and stabilizing ligand of the enzyme (72). Thus, the substrate is bound in a refoldable state. The overall picture that emerges is that α-Hsps act as very efficient molecular sponges that collect and immobilize denatured proteins on their way to irreversible aggregation. The storage of up to equimolar amounts of partially folded polypeptides provides a large reservoir of protein material that can be recycled under permissive conditions.

The chaperone action of α-Hsps described above is generally believed to be ATP independent (18, 137, 349). The addition of ATP did not enhance the chaperone activity of *E. coli* IbpB, *Mycobacterium tuberculosis* Hsp16.3, murine Hsp25, or pea Hsp18.1 and Hsp17.7 (44, 72, 174, 341). On the other hand, it has been proposed that the cellular ATP concentration modulates the chaperoning efficiency of certain α-Hsps. Light scattering of CS was suppressed by human αB-crystallin twice as efficiently in the presence of 3.5 mM ATP (216). Nonhydrolyzable ATP analogs had no stimulatory effect. Tryptophan fluorescence studies and mass spectrometry of tryptic digests of the chaperone demonstrated ATP-dependent structural modifications in the α-crystallin domain, which might influence its functionality (216, 217). ATP-enhanced chaperone activity of αB-crystallin was independently confirmed in experiments with the bovine protein (297). Parallel measurements performed with tobacco Hsp18 showed that ATP had an adverse effect; it reduced chaperone activity in the CS protection assay in a concentration-dependent manner. Again, conformational changes seem to be responsible for the ATP effect. In the presence of ATP, bis-ANS labeling of Hsp18 was reduced. Moreover, quenching of intrinsic tryptophan fluorescence sug-

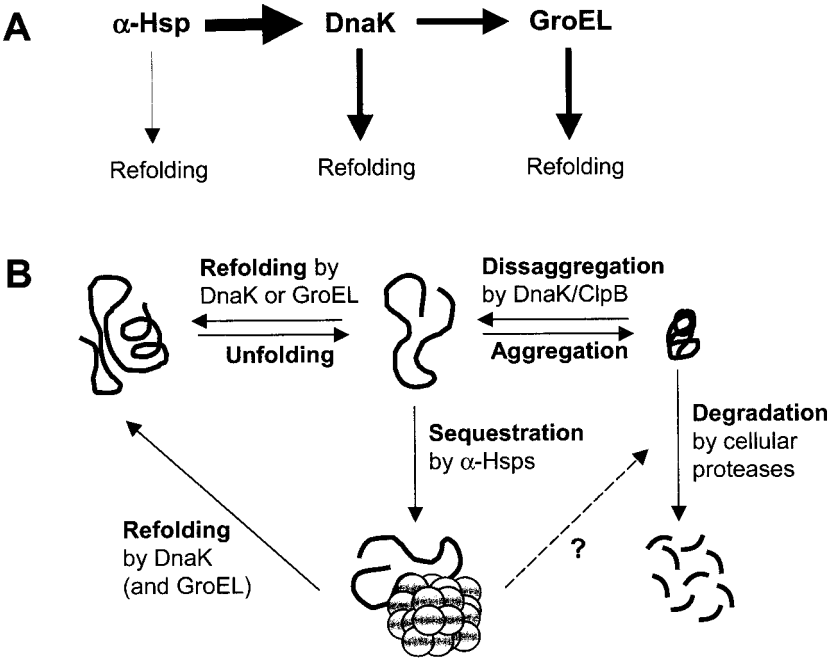

FIG. 6. Function of α-Hsps in a multichaperone network. (A) Sequential transfer of α-Hsp-bound substrates for further processing. (B) Schematic representation of the cellular multichaperone network. For details, see the text.

gested that Hsp18 has a more compact conformation in the presence of ATP. Whether these observations are of physiological relevance is a matter of speculation. Incidentally, the ATP concentration in the eye lens is higher than in most other tissues. Thus, stimulation of α-crystallin by ATP might be important for optimal chaperone activity in the lens (216). Abundant ATP in the plant cytosol is indicative of the absence of stress, which would make the action of α-Hsps obsolete. Under severe stress conditions when the ATP concentration drops, chaperones of the Hsp70 and Hsp60 families will not function efficiently. Simultaneous activation of α-Hsps would provide an elegant mechanism to coordinate the activity of ATP-dependent and ATP-independent chaperones by the availability of the nucleotide (297).

POSITION OF α-HEAT SHOCK PROTEINS IN A MULTICHAPERONE NETWORK

What is a cell going to do with the reservoir of folding-competent polypeptides that are deposited on the surface of α-Hsps? Although release and reactivation of bound substrates has been described, it is not a very productive process (72, 138, 216, 297, 341). When rabbit reticulocyte lysate, a rich source of chaperones, was added to luciferase complexed with either pea Hsp18.1 or bovine α-crystallin, enzyme activity was restored much more efficiently (175, 346). The stimulatory effect was strictly dependent on ATP. Since wheat germ extract had a similar, albeit weaker, effect on reactivation of Hsp18.1-bound luciferase with respect to that of rabbit reticulocyte lysate, it was proposed that a widely conserved ATP-dependent chaperone would assist in refolding (175). The first hint that this component might be Hsp70 (DnaK) came from the finding that addition of this chaperone together with ATP enhanced reactivation of Hsp25-bound CS (72). The observation that an *E. coli ibpAB* mutation exerted a deleterious growth defect at 42°C only in combination with a mutation in *dnaK* (*dnaK756*) but not with a mutation in *groESL* (*groES30*) implied a close cooperation with the DnaK machinery but not with GroESL in vivo (319). The structure of HSGs with α-Hsps in their inner core and Hsp70 on the surface is a further indication that the two chaperones work hand in hand (296).

Detailed biochemical studies confirmed that DnaK is the specific release factor for α-Hsp-bound substrates (176, 325, 341, 346). Refolding of heat-denatured MDH stabilized by IbpB was slow in the absence of other chaperones or in the presence of GroESL (341). Supplementation of MDH-IbpB with the DnaK system enhanced refolding significantly. Further recovery could be achieved by combining the DnaK and GroEL machineries. Gel filtration analyses demonstrated a sequential transfer of MDH from IbpB to DnaK and then to GroEL (341). Fig. 6A outlines the fate of substrate proteins bound to α-Hsps. It is assumed that only a small portion, if any, will refold spontaneously. The majority are being transferred to the DnaK machinery. One fraction will be refolded by DnaK, whereas another fraction must be passed on to GroEL before it can be refolded. In vitro, between 40 and 80% of MDH or lactate dehydrogenase was refolded by the combined action of IbpB, DnaK, and GroEL (341). The incomplete recovery may indicate that some enzyme escapes from chaperone-mediated protection by irreversible aggregate formation.

The position of α-Hsps in the context of a dynamic and synergistic multichaperone network in a stressed cell is depicted in Fig. 6B. Without α-Hsps, denatured proteins either can be refolded by the major chaperone machineries or will form insoluble aggregates destined for degradation. The pres-

ence of α-Hsps offers an alternative pathway that captures unfolding intermediates before they aggregate. The cell can make use of this large reservoir of folding-competent proteins whenever more suitable refolding conditions have been reestablished. It is interesting that IbpB displayed lower affinity to denatured MDH than did DnaK and GroEL (341). Hence, immediate refolding of denatured proteins by DnaK or GroEL seems to be the preferred option. The initial choice of chaperone probably depends on the availability. In this sense, α-Hsps serve as emergency chaperones under circumstances when DnaK and GroEL are occupied and/or when ATP becomes limiting. α-Hsps then reduce the flow of nonnative proteins toward aggregation and redirect them to the folding pathway.

The fact that DnaK rather than GroEL is required for immediate steps in further processing implies that the latter has no access to α-Hsp-bound proteins. It is unclear whether this is because GroEL encloses substrates in its central cavity and DnaK binds them at the surface or whether the unfolded proteins are in a conformation that cannot be handled by GroEL for other reasons. For example, two typical features of DnaK substrates are large size and local aggregation (67, 196, 211). Another unsolved puzzle is that some hyperthermophilic archaea lack an apparent *dnaK* homologue but nevertheless have a α-Hsp gene (192). Thus, substrate delivery pathways other than the one outlined above may occur in nature.

Bearing in mind the central position of α-Hsps in protein quality control (Fig. 6), it is perplexing that *E. coli ibpAB* mutants do not accumulate more aggregates after heat shock than the wild type does (319) or that the addition of IbpB to *E. coli* extracts does not prevent heat-induced protein aggregation (211). The deletion of *ibpA* and *ibpB* in *E. coli* did not produce a stress-sensitive mutant strain. However, overexpression of both genes increased the resistance to heat and superoxide stress (158). Moreover, thermotolerance of *E. coli* could be enhanced by the expression of various heterologous α-Hsps (216, 247, 301, 362). In contrast to *E. coli*, the deletion of *hsp16.6* in *Synechocystis* sp. strain PCC 6803 resulted in a clear growth defect after heat shock treatment (177, 178) and disruption of *hsp18* in *Streptomyces albus* showed that the gene is required for thermotolerance at extreme temperatures (275). Thus, there is cumulative in vivo evidence that α-Hsps do play a physiological role in the protection against adverse conditions. In general, it is difficult to ascertain the relative contribution of individual chaperones to protein folding. The simultaneous action of multiple chaperones hampers the design and interpretation of experiments in a cellular context. This is why in vitro studies of chaperone research often have been much more rewarding than in vivo experiments.

OTHER FUNCTIONS OF α-HEAT SHOCK PROTEINS

Members of the α-Hsp family have been implicated in an ever-growing number of cellular activities. The by no means complete list includes eye lens transparency (33); thermotolerance (169, 171, 177, 198, 248, 261, 270, 332, 362); resistance to apoptosis (10, 35, 45, 199–201); cytoskeleton modulation (20, 104, 172, 184, 207, 232); prevention of amyloid formation (165, 307); human diseases (48); various developmental processes in animals (11, 118, 181, 185, 203), plants (349), and bacteria (115); protection against oxidative stress (109, 259); desiccation tolerance (354); protection of the photosynthetic apparatus (113, 221) or of mitochondrial NADH:ubiquinone oxidoreductase (complex I) (68, 69); microbial pathogenicity (2, 54, 228, 283, 342, 363, 365); symbiotic and pathogenic plant-microbe interactions (16, 226); metabolic shift from acid to solvent production (245, 267); protection against acid shock (2, 95, 106); and sporulation (120). It is tempting to assume that the functional importance of α-Hsps in all these processes lies exclusively in the general protection of other proteins. Although this interpretation may be valid for most of the examples given above, there is increasing evidence that α-Hsps are concerned with functions other than chaperoning. The interaction between αB-crystallin and a proteosomal subunit might suggest that α-Hsps facilitate not only refolding but also selective degradation of target proteins (26) (Fig. 6).

Several studies demonstrate an interaction between α-Hsps and nucleic acids. HSGs in plants and mammals harbor untranslated mRNAs (149, 237). It is not clear whether α-Hsps directly bind RNA or whether the coassembly occurs via other proteins recruited into the HSG. A potential indirect role of α-Hsps in the transient storage and protection of mRNA during stress-induced translation arrest is supported by two lines of evidence. First, there is a specific association of several ribonuclear proteins and the heat shock transcription factor HsfA2 with HSGs (149, 268). Second, Hsp27 specifically interacts with the translation factor eIF4G and thereby inhibits protein synthesis under heat shock conditions, which are unfavorable for translation of most cellular mRNAs (53). Other reports show that α-crystallin directly binds single-stranded and double-stranded DNA (246, 290). Helices in α-crystallin have been suggested to play a role in DNA binding (25). Taken together, these observations suggest that α-Hsps may have largely unexplored functions in nucleic acid metabolism.

Recently, a potential link between α-Hsps and membrane functions has received growing attention. The occurrence of α-Hsps in the cell periphery has been reported repeatedly, for instance in *Stigmatella aurantiaca*, *Oenococcus oeni*, *M. tuberculosis*, and *E. coli* (61, 116, 142, 170, 173, 191). However, since α-Hsps are routinely detectable by standard two-dimensional gel electrophoresis (3, 95, 106, 205, 219, 226, 365), it appears that at least a major fraction is soluble and, if at all, only loosely attached to membranes. Studies with lens plasma membranes suggest that a subfraction of α-crystallin, once attached to the membrane, becomes irreversibly buried in the membrane bilayer (49). A critical role of α-Hsps in controlling the physical state of membranes has been established with Hsp17 (Hsp16.6) from *Synechocystis* strain sp. PCC 6803. This photosynthetic cyanobacterium forms irregular thylakoid membranes and evolves less oxygen when *hsp17* is inactivated (177, 178). The interpretation that Hsp17 is an important stabilizer of photosynthetic membranes is supported by the fact that the majority of heat-induced Hsp17 associates with thylakoid membranes (125). Altering the membrane physical state by administration of the fluidizer benzyl alcohol enhanced the thermosensitivity of *Synechocystis* cells. In parallel, the threshold temperature required for induction of heat shock genes, including *dnaK, groESL*, and *hsp17*, was reduced. The close correlation between membrane order and heat shock response led to the hypothesis that membranes act as cellular thermom-

eter from which stress is sensed and transduced into signaling pathways that activate heat shock gene expression (125, 344). Two features make membranes the prime candidate for monitoring thermal changes. First, they are among the first cellular structures that encounter external stress. Second, membrane fluidity is very sensitive to temperature variations, at both high and low temperatures (343, 344). Maintaining membrane integrity under stress conditions is an important job of Hsp17, as was shown by a combination of genetic and in vitro approaches. Fluorescence anisotropy measurements with isolated thylakoid membranes revealed that membrane fluidity was higher in preparations from an *hsp17* mutant than in wild-type membranes (325). The interaction between Hsp17 and large unilamellar vesicles made of synthetic or cyanobacterial lipids strongly increased the molecular order of the membranes. Penetration into the membrane hydrophobic core implies that Hsp17-membrane interactions are lipid mediated and are not due to membrane surface contacts. Lipid and substrate protein interaction seem to be mutually exclusive since the addition of lipids to an Hsp17-DnaK-GroEL refolding system strongly inhibited the reactivation of MDH (325). Under stress conditions, the cellular pool of α-Hsps is probably divided into a cytoplasmic subfraction responsible for regular chaperone activity and a membraneous subfraction involved in membrane stabilization. The most recent data demonstrate that specific lipid binding is not confined to cyanobacterial Hsp17 but also a feature of αB-crystallin (N. M. Tsvethova, I. Horvath, Z. Török, W. F. Wolkers, F. Tablin, E. Vierling, J. H. Gowa, and L. Vigh, submitted for publicatication). The precise nature of α-Hsp membrane interaction depends on the lipid composition and extent of fatty acid saturation. Most intriguingly, enhanced cell viability owing to the presence of α-Hsps has also been described during chilling (265, 266, 301). Membrane fluidity modulation by α-Hsps can fully explain a protective effect against both thermal extremes. While α-Hsps are able to rigidify membranes at high temperatures, they seem to fluidize saturated lipid species at low temperatures (Tsvethova et al., submitted).

In summary, α-Hsps seem to be very versatile protective agents. They play a dual role in protein and membrane protection and also might be involved in nucleic acid preservation. Chaperone research tends to overlook the fact that under stress conditions, the integrity of proteins is not the only thing at stake. Other macromolecules including nucleic acids and membranes will disintegrate at high temperatures if they are not protected immediately. In this context, it is interesting that GroEL has also been implicated in mRNA protection and membrane stabilization (59, 90, 326). Apparently, α-Hsps are not the only chaperones that help maintain the structural integrity of proteins, nucleic acids, and membranes.

CONCLUDING REMARKS

α-Hsps constitute the most divergent Hsp family. Despite their limited monomeric size, they socialize with each other, with other chaperones, and with diverse substrates through a large number of interactions, by which they serve to protect cellular macromolecules. Although the chaperone activity of α-Hsps was discovered almost a decade ago, many fundamental questions concerning this function remain unanswered. A strict correlation between oligomerization and chaperone activity is widely accepted. However, the underlying mechanistic details of this relationship are poorly understood. Moreover, the α-Hsp regions that contact substrate proteins are far from being defined, as are the recognition sites in unfolded proteins. Even more remains to be learned about the recently uncovered functions of α-Hsps. It is virtually unknown whether oligomerization is a prerequisite for interactions with nucleic acids or lipids. The α-Hsp sites interacting with these molecules still need to be explored. They are most probably different from those that contact unfolded proteins.

Revealing unifying concepts for the structure and function of α-Hsps is complicated by the fact that individual family members deviate substantially in their amino acid sequence and quaternary structure. Any isolated finding concerning one α-Hsp cannot necessarily claim to be true for others. Importantly, representative examples from archaea, bacteria, plants, and animals are now being investigated in order to arrive at a comprehensive picture. Only the concerted efforts of genetic, biochemical, biophysical, and structural studies will help unravel the general features of α-Hsps and the peculiarities of individual family members.

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